09-13-00

A / BOX SEQ

Please type a plus sign (+) inside this box → [+]

PTO/SB/05 (1/98)
Approved for use through 09/30/2000. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	4525US
First Inventor or Application Identifier	Strous et al.
Title	See 1 in Addendum
Express Mail Label No.	EL700253295US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. [ ] * Fee Transmittal Form (e.g., PTO/SB/17) (Submit an original, and a duplicate for fee processing)
2. [X] Specification [Total Pages 47] (preferred arrangement set forth below)
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (if filed)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) (35 U.S.C. 113) [Total Sheets 5]
4. Oath or Declaration [Total Pages]
   a. [ ] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d)) (for continuation/divisional with Box 17 completed) [Note Box 5 below]
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).
5. [X] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [ ] Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [X] Computer Readable Copy
   b. [X] Paper Copy (identical to computer copy)
   c. [X] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [ ] Assignment Papers (cover sheet & document(s))
9. [ ] 37 C.F.R.§3.73(b) Statement (when there is an assignee) [ ] Power of Attorney
10. [ ] English Translation Document (if applicable)
11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
12. [X] Preliminary Amendment
13. [X] Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
14. [ ] * Small Entity Statement(s) (PTO/SB/09-12) [ ] Statement filed in prior application, Status still proper and desired
15. [ ] Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. [ ] Other: ....

* A new statement is required to be entitled to pay small entity fees, except where one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No: PCT/NL99/00136

Prior application information: Examiner ______ Group / Art Unit: ______

18. CORRESPONDENCE ADDRESS

[X] Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label here) 24247 PATENT TRADEMARK OFFICE or [ ] Correspondence address below

Name	Allen C. Turner Trask Britt				
Address	P.O. Box 2550				
City	Salt Lake City	State	Utah	Zip Code	84110
Country	U.S.A.	Telephone	(801)532-1922	Fax	(801)531-9168

Name (Print/Type)	Kenneth B. Ludwig	Registration No. (Attorney/Agent)	42,814
Signature	Kenneth B. Ludwig	Date	09/12/00

Burden Hour Statement. This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Addendum

1. CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIBITORS OR RECEPTOR FRAGMENTS

Serial No. To Be Assigned

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Strous et al

Serial No.: To Be Assigned

Filed: September 12, 2000

For: CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIBITORS OR RECEPTOR FRAGMENTS

Examiner: To Be Assigned

Group Art Unit: To Be Assigned

Attorney Docket No.: 4075US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL 700253295US

Date of Deposit with USPS: September 12, 2000

Person making Deposit: Jared Turner

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Before examination and calculation of the filing fees, please amend the referenced application as follows:

IN THE SPECIFICATION:

Page 1, in the Title of the Invention, please change "INHIHITORS" TO --INHIBITORS--;

Page 1, line 1, at the beginning of the text insert

--Cross Reference to Related Application: This application is a continuation of International Application No. PCT/NL99/00136, filed on 12 March 1999 designating the United States of America, the contents of which are incorporated by this reference, which itself claims priority from European Patent Office Application Serial No. 98200799.9 filed 12 March 1998.--;

Page 1, line 1, before "The invention", please insert --Technical Field:--;

Page 1, line 2, please insert a comma after "example", and delete "to";

Page 1, line 3, please delete the comma after "proteins";

Page 1, line 6, before "For example", please insert --Background:--, and delete "For example";

Page 1, line 16, please delete the comma after "part";

Page 1, line 22, please insert a comma after "to" and afer "example";

Page 2, line 5, please insert quotations around "Receptors";

Page 2, line 8, please replace the comma after "membrane" with a period, and change "for" to --For--;

Page 2, line 10, delete the comma after "membrane";

Page 2, line28, delete the comma after "transduced";

Page 3, line 6, please insert a comma after "initiated";

Page 3, line 8, please change "internalised" to --internalized--;

Page 3, line 17, please insert a comma after "not" and after "insignificantly";

Page 4, line 4, please change "up regulation" to --up-regulation--;

Page 4, line 6, please change "up regulation" to --up-regulation--;

Page 4, line 4, please change "up regulation" to --up-regulation--;

Page 4, line 13, please delete the comma after "counterproductive" and insert --because-- therefore;

Page 4, line 24, please insert

--Disclosure of the Invention--;

Page 4, line 34, please insert a comma after "with" and "to";

Page 5, line 1, please insert a comma after the second occurrence of "protein";

Page 5, line 6, please change "fructose-1, 6-bifosfatase" to --fructose-1, 6-diphosphatase--;

Page 5, line 13, please change "gluconeogenese" to --gluconeogenesis--;

Page 5, line 23, please insert a comma after "invention" and "example";

Page 5, line 29, please delete the comma and insert --since-- in place thereof;

Page 5, line 32, before "inhibiting" please insert --thus,--;

Page 6, line 34, please change "down regulation" to --down-regulation--;

Page 7, line 3, please insert a comma after the first occurrence of "hormone";

Page 7, line 15, please change "prostaglandines" to --prostaglandins--;

Page 7, line 24, please change "biphosphatase" to --diphosphatase--;

Page 8, line 10, please insert a comma after "to";

Page 8, line 25, please insert a comma after "transduction" and after "time";

Page 8, line 30, please change "down regulation" to --down-regulation--;

Page 9, line 18, please insert --(SEQ. ID. NO. 1)-- after "xEFIxxDx";

Page 9, line 21, please insert quotation marks around "Essentially corresponding";

Page 9, line 29, please insert --(SEQ. ID. NO. 1)-- after "xEFIxxDx";

Page 9, line 31, please insert --(SEQ. ID. NO. 2)-- after "D-D-S-W-V-E-F-I-E-L-D-I";

Page 9, line 32, please insert --(SEQ. ID. NO. 3)-- after "D-S-W-V-E-F-I-E-L-D";

Page 10, line 12, please change "receptor" to --receptors--;

Page 10, line 13, please change "receptor" to --receptors--;

Page 10, line 22, please change "prostaglandines" to --prostaglandins--;

Page 10, line 25, please change "prostaglandines" to --prostaglandins--;

Page 11, line 1, please insert --(SEQ. ID. NO. 7)-- after "CEEDFYR";

Page 11, line 1, please insert a --(SEQ. ID. NO. 1)-- after "xEFIxxDx";

Page 11, line 12, please insert a comma after "Also";

Page 11, line 13, please delete the comma after "compounds";

Page 11, line 14, please change "skiled" to --skilled--;

Page 11, line 19, please change "Peptide analogous" to --Analogous peptides--;

Page 11, line 11, please change the comma after "art" to a period, and please change "for' to --For--;

Page 11, line 30, please insert --(SEQ. ID. NO. 4)-- after "PQRL";

Page 11, line 34, please insert --(SEQ. ID. NO. 5)-- after "PQRL";

Page 11, line 36, please change "endocytose" to --endocytosis--;

Page 12, line 6, please insert a comma after "example" and change "for" to --by--;

Page 13, line 1, please insert --The term-- prior to "In conjunction", please insert quotations around "In conjunction", please insert a comma after "conjunction", please insert --as used-- prior to "herein"; please insert a comma after "herein"; and please change "meaning" to --means--;

Page 13 , line 19, please change "down regulation" to --down-regulation--, and change "to" to --from--;

Page 13, line 20, please change "happen" to --happening--;

Page 13, line 23, please insert a comma after "Also";

Page 13, line 27, please insert --(SEQ. ID. NO. 6)-- after "GPDE";

Page 13, line 29, please change "accoprding" to --according--;

Page 14, line 10, please change "prostaglandines" to --prostaglandins--;

Page 14, line 34, please change "dimerisation" to --dimerization--;

Page 15, line 21, please change "to" to --with--;

Page 15, line 26, please change the comma after "respectively to a period; please change "for" to --For--, please insert a comma after "example", and please insert --a peptide or peptide analogue-- prior to "comprising";

Page 15, line 27, please insert --(SEQ. ID. NO. 7)-- after "FYR", and please insert --(SEQ. ID. NO. 1)-- after "xEFIxxDx";

Page 15, line 28, please insert a comma after "composition" and after "example";

Page 15, line 32, please change "to" to --with--;

Page 16, line 21, please change "Down regulation" to --Down-regulation--;

Page 16, line 25, please insert a comma after the second occurrence of "ligand";

Page 17, line 15, please insert a comma after "machinery";

Page 17, line 29, please insert a comma after “period”;

Page 17, line 31, please insert --the-- after “remove”;

Page 18, line 23, please change “visualised” to --visualized--;

Page 18, line 34, please insert a comma after “proteasome”;

Page 19, line 11, please insert a comma after “system”;

Page 19, line 24, please insert a comma after “reticulum”;

Page 19, line 26, please insert a comma after “cases”;

Page 19, line 31, please change “multidrug” to --multi-drug--;

Page 20, line 1, please insert a comma after “that”;

Page 20, line 2, please insert a comma after “event”;

Page 20, line 21, please change “downregulation” to --down-regulation--;

Page 20, line 30, please insert a comma after “Apparently”;

Page 21, line 14, please delete the comma after “receptor”;

Page 21, line 19, please insert a comma after “activity”;

Page 21, line 26, please insert a comma after “treatment”;

Page 21, line 29, please insert a comma after “K”;

Page 21, line 32, please insert a comma after “receptor)” and change “in” to --occur with--;

Page 22, line 1, please insert a comma after “site”;

Page 22, line 15, please insert a comma after “control”;

Page 22, line 26, please insert a comma after “vehicle”;

Page 22, line 30, please change “solubilisation” to --solubilization--;

Page 24, line 11, please change “visualised” to --visualized--;

Page 27, line 1 of Table 1, please insert --(SEQ. ID. NO. 8)-- after “SWVEFIELDI”;

Page 27, line 2 of Table 1, please insert --(SEQ. ID. NO. 9)-- after “LWVEFIELDI”;

Page 27, line 3 of Table 1, please insert --(SEQ. ID. NO. 10)-- after “LLVEYLEVDD”;

Page 27, line 4 of Table 1, please insert --(SEQ. ID. NO. 11)-- after “LLVEFLENDD”;

Page 27, line 5 of Table 1, please insert --(SEQ. ID. NO. 12)-- after “DNVDYLTRDW”;

Page 27, line 6 of Table 1, please insert --(SEQ. ID. NO. 13)-- after “QAAEYLRSET”;

Page 27, line 7 of Table 1, please insert --(SEQ. ID. NO. 14)-- after "IDAEYISAER";

Page 27, line 8 of Table 1, please insert --(SEQ. ID. NO. 15)-- after "LKGEFIWVDG";

Page 27, line 9 of Table 1, please insert --(SEQ. ID. NO. 16)-- after "YGSEYINLDG";

Page 27, line 10 of Table 1, please insert --(SEQ. ID. NO. 17)-- after "SEGEYIPLDQ";

Page 27, line 11 of Table 1, please insert --(SEQ. ID. NO. 18)-- after "DGHEYIYVDP";

Page 27, line 12 of Table 1, please insert --(SEQ. ID. NO. 19)-- after "DGHEYIYVDP";

Page 27, line 13 of Table 1, please insert --(SEQ. ID. NO. 20)-- after "DNFEYLTRDS";

Page 27, line 14 of Table 1, please insert --(SEQ. ID. NO. 21)-- after "KIFEYLRRDT";

Page 27, line 15 of Table 1, please insert --(SEQ. ID. NO. 22)-- after "SLQEYLQNDT";

Page 27, line 16 of Table 1, please insert --(SEQ. ID. NO. 23)-- after "TELEYLGPDE";

Page 27, line 17 of Table 1, please insert --(SEQ. ID. NO. 24)-- after "NQEEYLRYDS";

Page 27, line 18 of Table 1, please insert --(SEQ. ID. NO. 25)-- after " ENPEYLGLDV";

Page 27, line 19 of Table 1, please insert --(SEQ. ID. NO. 26)-- after "RLKEYLAGDV";

Page 27, line 20 of Table 1, please insert --(SEQ. ID. NO. 27)-- after "LYDKFLTLEH";

Page 27, line 21 of Table 1, please insert --(SEQ. ID. NO. 28)-- after "EQLEYLSYDA";

Page 27, line 22 of Table 1, please insert --(SEQ. ID. NO. 29)-- after "PEGEFLPLDQ";

Page 27, line 23 of Table 1, please insert --(SEQ. ID. NO. 30)-- after "SDSEFLLPDT";

Page 27, line 24 of Table 1, please insert --(SEQ. ID. NO. 31)-- after "SALDFIRRES";

Page 27, line 25 of Table 1, please insert --(SEQ. ID. NO. 32)-- after "AHNEYLVSEI";

Page 27, line 26 of Table 1, please insert --(SEQ. ID. NO. 33)-- after "VTLDFLDAEL";

Page 27, line 27 of Table 1, please insert --(SEQ. ID. NO. 34)-- after "EISDFLRYEL";

Page 27, line 28 of Table 1, please insert --(SEQ. ID. NO. 35)-- after "SAKDYIYQDS";

Page 27, line 29 of Table 1, please insert --(SEQ. ID. NO. 36)-- after "; YQQDFFPKEA";

Page 27, line 30 of Table 1, please insert --(SEQ. ID. NO. 37)-- after " SKLOYILAQI";

Page 27, line 31 of Table 1, please insert --(SEQ. ID. NO. 38)-- after "TPLNYILLNL";

Page 27, line 32 of Table 1, please insert --(SEQ. ID. NO. 39)-- after "TSVDLLDINV";

Page 27, line 33 of Table 1, please insert --(SEQ. ID. NO. 40)-- after "GTPDYIAPEI}";

Page 27, line 34 of Table 1, please insert --(SEQ. ID. NO. 41)-- after "GTPEYLAPEI}";

Page 27, line 35 of Table 1, please insert --(SEQ. ID. NO. 42)-- after "LYFEYLDKDL";

Page 27, line 36 of Table 1, please insert --(SEQ. ID. NO. 43)-- after "LVFEYLDSDL";

Page 27, line 37 of Table 1, please insert --(SEQ. ID. NO. 44)-- after "IGADFLTKEV";

Page 27, line 38 of Table 1, please insert --(SEQ. ID. NO. 45)-- after "IGVEFLNKDL";

Page 27, line 39 of Table 1, please insert --(SEQ. ID. NO. 46)-- after "ISVEFLVLDS";

Page 27, line 40 of Table 1, please insert --(SEQ. ID. NO. 47)-- after "SDIDFLIEEI";

Page 28, line 1 of Table 1, please insert --(SEQ. ID. NO. 48)-- after "AIGEFILVDK";

Page 28, line 2 of Table 1, please insert --(SEQ. ID. NO. 49)-- after "QKQEYKTLEY";

Page 2, line 3 of Table 1, please insert --(SEQ. ID. NO. 50)-- after "PPPxY"; and

Please insert the enclosed paper copy of the SEQUENCE LISTING at pages 31 through 42 of the application.

IN THE CLAIMS:

1. (Amended) A method for controlling or up-regulating the availability or activity of a protein comprising regulating binding of [the] a ubiquitin[/]-proteasome system at a ubiquitin[/]-proteasome binding site of said protein.

2. (Amended) [A]The method according to claim 1, wherein said ubiquitin-proteasome binding site comprises [the] an amino acid sequence motif xEFIxxDx (SEQ. ID. NO. 1)[or a sequence essentially corresponding thereto], wherein D is [the amino acid] aspartic acid, E is [the amino acid] glutamic acid, F is [the amino acid] phenylalanine, I is [the amino acid] isoleucine and X is any other amino acid.

3. (Amended) A method for controlling the [availability and/or] signal transduction capability of a cell surface receptor comprising providing an inhibitor capable of inhibiting proteolytic cleavage of said receptor.

4. (Amended) [A]The method according to claim 3 wherein said inhibitor is capable of inhibiting

proteolytic cleavage of an intra-cellular part of said receptor.

5. (Amended) [A]The method according to claim 3, wherein said inhibitor is capable of inhibiting proteolytic cleavage of an intra-cellular part of said receptor.

6. (Amended) [A]The method according to [anyone of] claim[s] 3 [to 5], wherein said receptor is a hormone receptor[, preferably selected from a group consisting of amino acid derivative, prostaglandine, peptide or protein hormone receptors].

7. (Amended) [A]The method according to claim 6, wherein said receptor is a growth hormone receptor.

8. (Amended) [A]The method according to claim[s] 1, [or 2] wherein said protein is a transport protein.

9. (Amended) [A]The method according to claim 8, wherein said transport protein is Glut4 insulin regulated glucose transporter.

10. (Amended) An inhibitor for regulating the availability or activity of a protein, said inhibitor comprising a [(poly)]polypeptide [or (poly)peptide analogue or mimeticum] that [is derived from, competes with, or binds to an amino acid sequence located at or around a] interferes with ubiquitin[/]-proteasome system regulation of cell surface receptors of a cell [binding site located in a protein].

11. (Amended) [A] The [(poly)peptide or (poly)peptide analogue or mimeticum] inhibitor according to claim 10, wherein said polypeptide interferes with said ubiquitin-proteasome system by binding to a ubiquitin-proteasome system binding site [comprises the] comprising an amino acid sequence motif xEFIxxDx (SEQ. ID. NO. 1)[or a sequence essentially corresponding thereto],

wherein D is [the amino acid] aspartic acid, E is [the amino acid] glutamic acid, F is [the amino acid] phenylalanine, I is [the amino acid] isoleucine and x is any other amino acid.

12. (Amended) The method according to claim 3, wherein [An] said inhibitor is capable of inhibiting proteolytic cleavage of a cell surface receptor [for use in a method according to anyone of claims 1 to 7].

13. (Amended) The method according to claim 12, wherein said [An] inhibitor [according to claim 12 which] is capable of inhibiting proteolytic cleavage of [the] an intra-cellular part of said receptor.

14. (Amended) The method according to claim 13, wherein said [An] inhibitor [according to claim 13] is selected from the group of proteasome inhibitors[, such as] consisting of MG132, carboxybenzyl-leucyl-leucyl-leucinal, lactacystin, carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone [or] and the β-lacton form of lactacystin.

15. (Amended) The method according to claim 13, wherein said [An] inhibitor [according to claim 13 comprising] comprises a [(poly)]polypeptide [or (poly)peptide analogue or mimeticum] that is derived from, competes with, or binds to an amino acid sequence located at or around a [ubiquitin and/or] ubiquitin[/]-proteasome system binding site located in [the] an intra-cellular part of a cell-surface receptor.

16. (Amended) The method [An inhibitor] according to claim 15 wherein, said ubiquitin-proteasome system binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto, wherein D is [the amino acid] aspartic acid, E is [the amino acid] glutamic acid, F is [the amino acid] phenylalanine, I is [the amino acid] isoleucine and X is any other amino acid.

17. (Amended) The method according to claim 16, [An inhibitor according to claim 16] wherein

said ubiquitin-proteasome system binding site comprises [the] an amino acid sequence selected from the group consisting of DDSWVEFIELDI (SEQ. ID. NO. 2) [or] and DSWVEFIELD (SEQ. ID. NO. 3).

18. (Amended) The method according to claim 12, wherein said [An] inhibitor [according to claim 12] is capable of inhibiting proteolytic cleavage of extra-cellular part of said receptor.

19. (Amended) The method [An inhibitor] according to claim 18, wherein said extra-cellular part comprises an approximately 60 kDa fragment of an extra-cellular domain of the growth hormone receptor.

20. (Amended) The method [An inhibitor] according to claim 18 [or 19], wherein said inhibitor [comprising] comprises a [(poly)]polypeptide [or (poly)peptide analogue or mimeticum] that is derived from, competes with or binds to an amino acid sequence located at or around a proteolytic cleavage signal site located in an extra-cellular part of said receptor.

21. (Amended) The method [An inhibitor] according to claim 20, wherein said cleavage signal site comprises [that] the amino acid sequence CEEDFYR (SEQ. ID. NO. 7)[or a sequence essentially corresponding thereto].

22. (Amended) The inhibitor according to claim 10, wherein said [A (poly)peptide or (poly)]polypeptide [analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a ubiquitin and/or ubiquitin/proteasome system] interferes with said ubiquitin-proteasome system by binding to a ubiquitin-proteasome system binding site located in the intra-cellular part of a cell-surface receptor.

23. (Amended) The [A (poly)peptide or (poly)peptide analogue or mimeticum] inhibitor according to claim 22, wherein said binding site comprises [the] an amino acid sequence motif xEFIxxDx

(SEQ. ID. NO. 1)[or a sequence essentially corresponding thereto], wherein D is [the amino acid] aspartic acid, E is [the amino acid] glutamic acid, F is [the amino acid] phenylalanine, I is [the amino acid] isoleucine and x is any other amino acid.

24. (Amended) The inhibitor according to claim 10, wherein said [A (poly)peptide or (poly)]polypeptide [analogue or mimeticum that is derived from, competes with or binds to] interferes with said ubiquitin-proteasome system by binding to an amino acid sequence located at or around a proteolytic cleavage signal site located in [the] an extra-cellular part of a receptor.

25. (Amended) The inhibitor [A (poly)peptide or (poly)] peptide [analogue or mimeticum] according to claim 24, wherein said cleavage signal site comprises [the] an amino acid sequence CEEDFYR (SEQ. ID. NO. 7) [or a sequence essentially corresponding thereto].

26. (Amended) A pharmaceutical composition comprising an inhibitor according to [any of claims 12-21 or a (poly)peptide or (poly)peptide analogue or mimeticum according to any of] claim[s] 10[, 11, or 22-25].

28. (Amended) A pharmaceutical composition according to claim 27 for [administering] administration in conjunction with a hormone.

29. (Amended) The pharmaceutical composition according to claim 26, wherein [Use of an inhibitor according to any of claims 12 to 21 or a (poly)peptide or (poly)peptide analogue or mimeticum according to any of claims 10,11 or 22-25 for the production of a pharmaceutical composition] said inhibitor is used for controlling the availability and or signal transduction capability of a cell surface receptor.

30. (Amended) The pharmaceutical composition according to claim 29, wherein said [Use according to claim 29 for the production of a] pharmaceutical composition is used for regulating the

activity of a hormone.

31. (Amended) The pharmaceutical composition according to claim 29, [Use according to claim 29 or 30] wherein said pharmaceutical composition is administered in conjunction to the administration of said hormone.

32. (Amended) The pharmaceutical composition according to claim 29, wherein said pharmaceutical composition is used [Use according to any of claims 29 to 30 for the production of a pharmaceutical composition] for [the] treatment of muscle wasting.

33. (Amended) The [A] method according to claim 1, wherein, said regulating binding of the a ubiquitin-proteasome system at a ubiquitin-proteasome binding site of said protein comprises controlling or up-regulating [to control or up-regulate] hormone activity by using an inhibitor polypeptide which interferes with ubiquitin-proteasome system regulation of cell surface receptors of a cell [according to any of claims 12 to 21 or a pharmaceutical composition according to any of claims 26 to 28].

Please add the following new claims:

34. The method according to claim 6, wherein said hormone receptor is selected from the group consisting of amino acid derivatives, prostaglands, peptides or protein hormone receptors.

35. The inhibitor according to claim 10, wherein said polypeptide interferes with said ubiquitin-proteasome system regulation of cell surface receptors of a cell by inhibiting ligand-induced receptor uptake.

36. The inhibitor according to claim 10, wherein said polypeptide interferes with said ubiquitin-proteasome system regulation of cell surface receptors of a cell by inhibiting receptor degradation caused by endocytosis.

Remarks

The application is to be amended without prejudice or disclaimer as previously set forth. Primarily, the amendments are sought to conform the application to a form more consistent with Office practice and to remove multiple dependencies. The specification is to be amended to include a sequence listing. It is respectfully submitted that no new matter has been added by the amendments. However, if any questions remain after consideration of the instant amendments, the Office is kindly requested to contact applicants' attorney at the address or telephone number given herein.

Respectfully submitted,

Kenneth B. Ludwig

Kenneth B. Ludwig
Registration No. 42,814
Attorney for Applicants
TRASK BRITT
P. O. Box 2550
Salt Lake City, Utah 84110-2550
(801) 532-1922

Date: September 12, 2000

Enclosure: Preliminary Examination Report
Sequence Listing under 37 CFR 1.821 - 1.825

<u>PATENT</u>
Attorney Docket 4525US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL700253295US

Date of Deposit with USPS: September 12, 2000

Person making Deposit: Jared Turner

APPLICATION FOR LETTERS PATENT

for

CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIBITORS OR RECEPTOR FRAGMENTS

Inventors:
Gerardus Jacobus Antonius Maria Strous
Petrus Johannes Maria Van Kerkhof
Roland Marinus Theodorus Govers

Attorney:
Allen C. Turner
Registration No. 33,041
Kenneth B. Ludwig
Registration No. 42,814
TRASK BRITT
P.O. Box 2550
Salt Lake City, Utah 84110
(801) 532-1922

CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIHITORS OR RECEPTOR FRAGMENTS

The invention relates to the field of regulating metabolic processes, for example to regulating availability and/or activity of proteins, such as (cytosolic) transport proteins, enzymes and cytosolic or membrane bound receptor proteins.

For example, a receptor protein on the surface of a cell has a binding site with a high affinity for a particular signalling substance (a hormone, pheromone, neurotransmitter, etc.). The specific signalling substance is often referred to as the ligand, a substance that binds to or fits in a site, the ligand binding site. When the signalling substance binds to a receptor, a receptor-ligand complex initiates a sequence or cascade of reactions that changes the function of the cell. A cell surface receptor polypeptide typically comprises an extra-cellular part which comprises a binding site where the ligand can interact, a transmembrane part, that locates a receptor in the cell membrane, and an intracellular part that plays a role in transducing a signal further into the cell once a ligand has bound. A receptor polypeptide can span the cell membrane several times resulting in multiple extra- and intracellular domains.

The response of a cell or tissue to for example specific hormones is dictated by the particular receptors it possesses and by the intra- or inter-cellular reactions initiated by the binding of any one hormone to its receptor. One cell may have two or more types of receptors or various cell types may have different sets of receptors for the same ligand, each of which induces a different response. Or the same receptor may occur on various cell types, and binding of the same ligand may trigger a different response in each type of cells. Clearly, different cells respond in a variety of ways to the same ligand, depending on a receptor or its interaction with the cell.

A wide variety of receptors specific for a wealth of ligands exist. Examples can be found among ion-channels, such as Ca^+-channels, or Cl^--channels or Na^+ channels, glucose transporters; among immunoglobulin receptors, such as IgE receptors; among cytokine receptors; among multi-drug transporters, and so on. Receptors, as defined herein, relate to signal transducing molecules in the broadest sense. These transducing molecules include ion-pump like proteins, for example the above-mentioned ion channels that transport the ligand (here the ion) through the membrane, include receptors that bind to a ligand (eliciting a signal over the membrane) but that do not transport the ligand itself through the membrane, include transport proteins and include enzymes that act upon contact with a substrate. As an example, receptors having as ligand a hormone are herein discussed in more detail, however, physiological mechanisms regulating hormone receptor availability and signal transduction are also found among a large variety of receptors having another ligand.

Hormones reacting with cell surface receptors are of a varied nature. Typical examples are amino acid derivatives such as epinephrine or histamine, prostaglandines, various peptide hormones such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, EGF, and others.

These hormones all act by binding to their specific surface receptor, after which their specific signal is being transduced, directly or by an intracellular signaling substance (a second messenger), leading to the specific action that is required of the cell.

The amount of functional (hormone) receptor on the cell surface is not constant. A receptor level is modulated up (up regulation) or down (down-regulation), permitting the cell to respond to small changes in the hormone (ligand) level. The number of cell surface receptors is often down-regulated by

endocytosis, whereby the sensitivity of the cell for the specific hormone (ligand) is reduced.

In general, when a ligand (hormone) binds to its receptor and results in a ligand-receptor complex, two phenomena occur. On the one hand, the signal transduction cascade is initiated while on the other hand the ligand-receptor complexes are brought in the cell by receptor mediated endocytosis and the internalised ligand (hormone) is degraded. Internalisation and degradation most likely terminate the hormone signal.

Some receptors recycle to the cell surface by exocytosis, however, even if they do, often a substantial fraction will be in the internal membrane compartments at any one time. Fewer receptors will be on the cell surface, available to bind extra-cellular hormone. Other receptors get degraded by proteolytic cleavage processes in the cell and thus do not or only insignificantly recycle to the cell surface, again reducing the number of available receptors on the cell surface.

Another way by which receptor availability on the cell surface is down-regulated is by removal, for example by specific proteolysis, of the extra-cellular part that comprises parts of the binding site of a receptor. Such removal is in essence a physiological mechanism that serves to refresh the available receptors and replace them with new ones, however, it again is a factor in reducing receptor availability.

As a consequence of fewer functional receptors being available on the cell surface, the hormone concentration necessary to induce the physiological response is higher and the sensitivity of the cell to the hormone is reduced. The susceptibility of a cell or tissue to the action of a hormone is thus among others dependent on the number of functional receptors present at any given time on the surface of a cell. Even when ligands are circulating at a high concentration,

these cannot result in sufficient activation when not enough receptors are present.

Many hormonal related or other diseases would benefit from an up regulation of hormone or ligand activity. In hormonal dysfunctioning, one often attempts to achieve such up regulation simply by treating a patient with exogenous hormones, however, as explained above, such a treatment may not be effective due to the fact that the number of available surface receptors for that hormone are too low. This is often aggravated by the fact that higher hormone concentrations enhance, by feed-back mechanisms, the further down-regulation of the specific receptor. Exogenous hormone therapy may then even be counterproductive, the patient becomes less susceptible to the hormone in question.

There is thus a need for pharmacological tools or medication that can up- or down-regulate the presence or signal-transduction of ligand-specific proteins such as transport proteins, enzymes, cytosolic receptors or receptors on the surface of cells, for example to make a patient better responding to hormonal therapy or to provide the cells of said patient with a higher sensitivity to an endogenous hormone or other ligand.

The invention provides a method for controlling or up-regulating the availability or activity of a protein comprising regulating or mimicking binding of the ubiquitin/proteasome system at the ubiquitin/proteasome system binding site of said protein, said protein for example being a transport protein, an enzyme, a cytosolic receptor protein or cell-surface receptor protein.

The invention also provides a (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a ubiquitin/proteasome system binding site located in a protein, said protein for example being a transport

protein, an enzyme, a cytosolic receptor protein or cell-surface receptor protein. Herewith the invention provides means for applying a method for controlling or up-regulating the availability or activity of a cytosolic receptor protein or transport protein, such as rab7 or rab9 GTPases or Glut4, an enzyme, such as fructose-1,6-bifosfatase (FBPase) or a cell-surface receptor, such as a growth hormone receptor (GHR).

Through binding at said binding site, the ubiquitin/proteasome system plays a regulating role with a large number of important metabolic processes. Anabolic regulating mechanisms (i.e. acting through GHR, FBPase, Glut4, etc., for example involved in gluconeogenese) are in this way kept in balance by the catabolic acting ubiquitin/proteasome system. Increased activity of catabolic processes (for example through corticosteroid action) causes down-regulation of anabolic mechanisms.

In a preferred embodiment the invention provides a (poly)peptide or (poly)peptide analogue or mimeticum according to the invention wherein said binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto.

The invention for example provides a method for controlling or up-regulating the availability and/or signal transduction capability of a cell surface receptor comprising providing an inhibitor capable of inhibiting proteolytic cleavage or truncation of said receptor. Said proteolytic cleavage or truncation of a receptor provides the basis for down-regulation, cleavage at either the extra-cellular or intra-cellular part of a receptor renders the receptor unavailable for ligand interaction and/or signal transduction; inhibiting said proteolytic cleavage or truncation of a receptor therefor provides a longer or more dense receptor availability at the cell surface and provides better signal transduction to the cell. Also, an increased transport of a transporter/receptor from the intracellular

compartments to the cell-surface and/or a decreased transport of a transporter/receptor from the cell surface to intracellular compartments is now induced, whereby the average time that a transporter/receptor stays at the surface of the cell is increased.

The invention provides a method wherein said inhibitor is capable of inhibiting proteolytic cleavage or truncation of an extra-cellular part of said receptor. An example of such extra-cellular cleavage is given in the experimental part of this description wherein such truncation or cleavage comprises removal of a 60kD fragment comprising a soluble growth hormone (GH) binding-protein from the extra-cellular domain of the growth hormone receptor (GHR) from a 70kD fragment comprising a transmembrane and inter-cellular part of said receptor. Said proteolytic cleavage or truncation preferably occurs at or around the amino acid sequence CEEDFYR found for example in the growth hormone receptor.

A preferred embodiment provided by the invention is a method wherein said inhibitor is capable of inhibiting proteolytic cleavage or truncation of an intra-cellular part of said receptor. An example of such intra-cellular cleavage is given in the experimental part of this description wherein such truncation or cleavage preferably is initiated by the action of the ubiquitin conjugating system, after which the proteasome can initiate proteolytic cleavage or truncation. We have detected that this ubiquitin/proteasome system is involved in ligand-induced degradation of cell surface receptors. Binding of hormone initiates signal transduction and at the same time the ubiquitin/proteasome system is activated and leads to endocytosis and/or degradation of a receptor. Inhibiting the ubiquitin/proteasome system, for example by preventing ubiquitin and/or the ubiquitin conjugating system to bind to its intra-cellular binding site at a receptor, prevents this down regulation to happen and leads to prolonged or more intense signal transduction, thus increasing the ligand activity independent of increased

ligand concentration. According to preferred embodiments of the invention said ligand is a hormone (see examples above), preferably a growth hormone or said receptor is a hormone receptor (of which various examples are given throughout the description), preferably a growth hormone receptor.

The invention provides means and methods to control or up-regulate exogenous and/or endogenous ligand (for example a hormone) activity by using specific inhibitors, or inhibiting reagents such as peptides or peptide analogues, that control (inhibit, counteract or modify) down-regulation of a cell surface receptor. The invention provides a method or an inhibitor for controlling or up-regulating for example cell surface receptors for hormones such as tyroxine, amino acid derivatives such as epinephrine, histamine or glutamine, prostaglandines, peptide or protein hormones such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, leptin, nerve growth factor, EGF, FAS, or that are cytokines. Also, transport proteins, such as calcium-, sodium-, potassium, chloride-, proton-channel proteins, and glucose transport proteins (for example Glut4) and cytosolic (non-membrane) proteins such as small GTP-binding protein Rab 7 or 9 and fructose-1,6-biphosphatase (FBPase) are receptors or (transport) proteins (examples are CFTR, aquaporins, ENAC, see also Table 1) that can now be controlled or up-regulated by a method provided by the invention.

The invention also provides an inhibitor capable of inhibiting proteolytic cleavage or truncation of a cell surface receptor, for example for use in a method provided by the invention. One embodiment of the invention is an inhibitor which is capable of inhibiting proteolytic cleavage of the ligand binding site of the extra-cellular part of said receptor. An example of such an inhibitor provided by the invention is an inhibitor which is capable of inhibiting removal of a 60kD fragment comprising a soluble growth

hormone (GH) binding-protein from the extra-cellular domain of the growth hormone receptor (GHR) from a 70kD fragment comprising a transmembrane and an intra-cellular part of said receptor. Said inhibitor can be a inhibitor of proteolytic cleavage per se, being broadly specific for a variety of proteolytic enzymes, or being narrowly specific for the distinct proteolytic enzyme or enzymes involved. A preferred embodiment of an inhibitor provided by the invention comprises a (poly)peptide or (poly)peptide analogue that is derived from, competes with or binds to an amino acid sequence located at or around a proteolytic cleavage signal site located in the extra-cellular part of a cell-surface receptor. An example of such a cleavage signal site comprises the amino acid sequence CEEDFYR (or a sequence essentially corresponding thereto), found in the extra-cellular polypeptide part of a growth hormone receptor.

Another inhibitor according to the invention is an inhibitor which is capable of inhibiting ligand-induced receptor uptake and/or degradation by endocytosis of said receptor, for example an inhibitor which is capable of inhibiting ligand-induced receptor uptake and/or degradation by the ubiquitin/proteasome system. We have detected that the ubiquitin/proteasome system is involved in ligand-induced degradation of cell surface receptors. Binding of ligand to a receptor initiates signal transduction and at the same time the ubiquitin and/or ubiquitin/proteasome system is activated by binding to the intra-cellular part of the receptor which leads to endocytosis and/or proteolytic cleavage or truncation of a receptor. Inhibiting the ubiquitin/proteasome system prevents this down regulation to happen and leads to prolonged or more intense signal transduction, thus increasing the hormonal activity independent of increased hormone concentration.

The invention provides an inhibitor capable of inhibiting proteolytic cleavage of the intra-cellular part of a cell surface receptor. Said inhibitor can be a inhibitor of

proteolytic cleavage per se, being broadly specific for proteolytic enzymes, or being narrowly specific for the distinct proteolytic enzyme or enzymes involved, or can be an inhibitor of ubiquitin binding, whereby further proteolytic cleavage is prevented. A preferred embodiment of an inhibitor provided by the invention is a proteasome inhibitor, for example selected from the group of proteasome inhibitors, such as MG132, carboxybenzyl-leucyl-leucyl-leucinal, lactacystin, carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone or the β-lacton form of lactacystin.

Another preferred embodiment of an inhibitor provided by the invention comprises a (poly)peptide or (poly)peptide analogue that is derived from, competes with or binds to an amino acid sequence located at or around a ubiquitin and/or ubiquitin/proteasome-system binding site located in the intra-cellular polypeptide part of a cell-surface receptor. An example of such a binding site comprises the amino acid sequence motif xEFIxxDx, or a sequence essentially corresponding thereto, found in the intra-cellular polypeptide part of a receptor.

Essentially corresponding means herein that the amino acid motif provided by the invention relates to a variety of specific amino acid sequences. For example, the amino acid E (glutamic acid) in the motif can be replaced by the like amino acid D (aspartic acid), F (phenylalanine) can be replaced by Y (tyrosine), I (isoleucine) by L (leucine), V (valine) or F (phenylalanine), S (serine) by T (threonine), or D by E. Examples of amino acid sequences that are essentially corresponding to the amino acid motif xEFIxxDx are listed in Table 1. A further detailed example is the amino acid sequence D-D-S-W-V-E-F-I-E-L-D-I or D-S-W-V-E-F-I-E-L-D, located, for example, at a distance of about 50 amino acid residues from the plasma membrane in the intracellular part of the growth hormone receptor. Conversion of each of the 12 amino acids in the motif into alanine residues showed that each amino acid contributes (albeit with varying

intensity) to the possible interaction between hormone receptor and ubiquitin or the ubiquitin/proteasome-system, and thus to the mechanism of the ubiquitin/proteasome-dependent endocytosis and cleavage of a receptor. Experiments with ^{125}I-labelled growth hormone demonstrated that in particular the conversions of S^{323}->A, E^{326}->A, F^{327}->A, I^{328}->A and D^{331}->A in the growth hormone receptor affected endocytosis of growth hormone (100% effects), while conversion of the amino acid positions D^{321}, D^{322}, W^{324}, E^{329}, L^{330}, I^{332} into alanine resulted in intermediate effects; mutation V^{325}->A did not affect the interaction. Growth hormone receptor with tail truncations of up to amino acid D^{334} functioned as wild-type receptor with respect to the interaction of the presently invented system. Thus, the above mentioned amino acid motif or variations therein, are instrumental for interaction with ubiquitin and/or with the ubiquitin/proteasome system in endocytosis and/or cleavage of a receptor.

The invention provides an inhibitor that is capable of inhibiting down-regulation of the cell surface receptor of a hormone wherein said hormone is selected from a group composed of amino acid derivatives, prostaglandines, peptide or protein hormones and cytokines. Hormones such as tyroxine, amino acid derivatives such as epinephrine, histamine or glutamine, prostaglandines, peptide- or protein-hormones such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, leptin, nerve growth factor, EGF, FAS, or that are cytokines. Also, transport proteins, such as glucose transporter, calcium-, sodium-, potassium, chloride- and proton-channel proteins are receptors (examples are CFTR, aquaporins, ENAC) that can now be controlled or up-regulated by an inhibitor provided by the invention. The invention provides a (poly)peptide or (poly)peptide analogue or mimeticum derived from an amino acid sequence that corresponds to the amino acid motif

CEEDFYR, or xEFIxxDx or to a motif essentially corresponding thereto. A (poly)peptide that competes, binds, or interacts in another way with such an amino acid sequence is herein also considered to be derived from an amino acid sequence corresponding to such a motif. Such a peptide is selected according to methods known by a person skilled in the art. It is to be expected that the conformation of a selected (poly)peptide is important for its reactivity. Appropriate conformational changes can be introduced in selected (poly)peptides by techniques known to the person skilled in the art, for example it is possible to introduce appropriate conformation by using di-sulfide bridges. Also other peptide sequences or compounds, mimicking the wanted conformation (mimetica) can be selected by a person skiled in the art. A suitable system to select a (poly)peptide is a system such as the PEPSCAN system, whereby interaction, such as competition or inhibition, is measured against sets of overlapping peptides chosen from the receptor's amino acid sequence. Peptide analogous, whereby specific amino acids are replaced by others, being either L-, or D-amino acids, are tested similarly. Other methods include replacement net scanning of selected peptide sequences, for example by replacing distinct amino acids by alanine, whereby crucial amino acids in the selected peptides are determined. (Poly)peptides can be made synthetically or via recombinant techniques. Suitable (poly)peptides for up-regulation of GH activity are derived for example from the amino acid sequence: SKQQRIKMLILPPVPVPKIKGIDPDLLKEGKLEEVNTILAIHDSYKPEFHSDDSWVEFIEL DIDEPDEKTEESDTDRLLSSDHEKSHSNLGVKDGDSGRTSCCEPDILETDFNANDIHEGTS EVAQPQRL found with the growth hormone receptor. An example of such a selected sequence comprises the amino acid sequence SWVEFIELDIDD or variations thereon, as for example shown in Table 1. Another example is the sequence KDGDSGRTSCCEPDILETDFNANDIHEGTSEVAQPQRL that comprises a signal site allowing the proteasome to stop, after which endocytose occurs.

Furthermore, the invention provides a pharmaceutical composition comprising an inhibitor that is capable of controlling the availability and/or signal transduction capability of a cell surface receptor or inhibiting down-regulation of a cell surface receptor according to the invention, for example for regulating the activity of a hormone. Such a pharmaceutical composition for example finds its use in treating patients with hormone deficiencies, such as growth hormone deficiencies. Yet another example of use of a pharmaceutical composition provided by the invention is for the treatment of patients that are suffering from (muscle) wasting that results from increased (muscle) protein degradation that is often seen after or during disorders such as renal tubular defects, uraemia, diabetes, Cushing's syndrome, cachexias seen with cancer or with eating disorders, after serious burns, during sepsis or AIDS, after stress, during and after immobilisation, during neuromuscular disease, and other conditions that alter protein degradation in cells such as muscle cells. Experimental animal models are available to study these and other related disorders. One can for instance use rats to study fasting, metabolic acidosis, kidney failure, muscle denervation, diabetes, thermal injury, endotoxaemia, bacteraemia, tumour development, glucocorticoid or thyroid hormone treatment and hyperthyroidism. Studies of such experimental models have indicated that the ubiquitin/proteasome pathway is activated in muscle and causes the loss of muscle mass (wasting) in these disorders. The invention provides the use of pharmaceutical compositions inhibiting this pathway and controlling or up-regulating the availability of hormone receptors, such as growth hormone receptors, thereby activating anabolic processes in the cells and decreasing the protein degradation seen with above described disorders related to muscle wasting.

Yet another embodiment of the invention is a pharmaceutical composition comprising an inhibitor provided by the invention which composition is administered in

conjunction with a hormone. In conjunction herein meaning that said composition is used or applied before, during or after hormonal treatment and up-regulates or modifies the activity of said hormone.

Yet another embodiment of a method provided by the invention is a method wherein up-regulation of hormone receptors is achieved via preventing ligand-induced receptor mediated uptake and degradation by endocytosis mediated via the intra-cellular part of a receptor. A preferred embodiment of the invention is a method whereby the ubiquitin/proteasome system that is involved in ligand-induced degradation of a hormone receptor is inhibited. We have detected that ubiquitin conjugation and/or subsequent proteasome action (the ubiquitin/proteasome system) is involved in ligand-induced degradation of cell surface receptors. Binding of hormone initiates signal transduction and at the same time the ubiquitin/proteasome system is activated and leads to endocytosis and/or degradation of a receptor. Inhibiting the ubiquitin/proteasome system prevents this down regulation to happen and leads to longer or higher signal transduction, thus increasing the hormonal activity independent of increased hormone concentration.

Also the invention provides a method for controlling or up-regulating the availability of the Glut4 insulin regulated glucose transporter (Glut4). Herein, interaction with the ubiquitin/proteasome system is regulated through binding at an amino acid motif TELEYLGPDE (see Table 1). Regulating said interaction allows for regulating endocytosis of Glut4. By down-regulating endocytosis of Glut4 with a method accoprding to the invention, the invetion provides a novel way of treating diabetes, type I as well as type II. High glucose plasma levels can now be reduced or tampered independent from regulating insulin or insulin-receptor levels.

The invention provides a method for controlling or up-regulating the availability of a large variety of cell surface receptors. It has now been found that the turn-over

of these receptors can be regulated by ubiquitin binding, for example in the ubiquitin/proteasome system. Inhibiting said system prevents or retards turn-over of said receptors and may lead to longer or higher signal transduction, thus increasing the hormonal activity independent of increased hormone concentration. The invention provides a method or an inhibitor for controlling or up-regulating for example cell surface receptors for hormones such as tyroxine, amino acid derivatives such as epinephrine, histamine or glutamine, prostaglandines, peptide or protein hormones such as glucagon, insulin, gastrin, secretin, ACTH, LH, FSH, TSH, TRH, LHRH, vasopressin, IGF-I or II, EGF, somatotropin (growth hormone), prolactin, erytropoietin, leptin, nerve growth factor, EGF, FAS, or that are cytokines. Also, transport proteins, such as calcium-, sodium-, potassium, chloride- and proton-channel proteins are receptors (examples are glucose transporters, CFTR, aquaporins, ENAC, see also Table 1) that can now be controlled or up-regulated by a method provided by the invention. The invention provides means and methods to control the action of the ubiquitin/proteasome system to up-regulate hormone activity by using specific inhibitors of proteasome action or by using reagents that compete for the ubiquitin/proteasome system recognition sites on a receptor or membrane channel.

An example of a method provided by the invention is given in the experimental part, where a method inhibiting the ubiquitin/proteasome system that is involved in ligand-induced degradation of a growth hormone receptor is further explained. The growth hormone receptor is important for normal growth and development in animals and humans. Biological effects include linear growth, lactation, nitrogen retention, lipolysis, diabetogenic-like effects, macrophage activation, and others. Binding of growth hormone induces dimerisation of two receptor polypeptides. This double binding initiates signal transduction and at the same time the ubiquitin/proteasome system is activated and removes a

considerable part of the cytosolic or intra-cellular tails of a receptor. This proteolytic event is an obligatory step in the cascade of reactions which lead to endocytosis and degradation of a receptor. The invention provides means and methods to control the action of the ubiquitin/proteasome system to up-regulate growth hormone activity by using inhibitors such as specific inhibitors of proteasome action or reagents that compete for the ubiquitin/proteasome system recognition sites on a receptor

The invention also provides the use of a proteasome inhibitor, for example MG132, carboxybenzyl-leucyl-leucyl-leucinal, lactacystin, carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone or the β-lacton form of lactacystin, for the production of a pharmaceutical composition. As an example the invention provides the use of such a proteasome inhibitor for the production of a pharmaceutical composition regulating the activity of a hormone, such as a growth hormone. Furthermore, the invention provides the use of such a proteasome inhibitor for the production of a pharmaceutical composition regulating the activity of a hormone wherein said composition is administered in conjunction to the administration of said hormone.

The invention also provides the use of a peptide or peptide analogue competing with and derived from an intra-cellular or extracellular protein part of a receptor respectively, for example comprising the amino acid motif CEEDFYR or xEFIxxDx for the production of a pharmaceutical composition for example for controlling the availability and/or signal transduction capability of a cell surface receptor or for regulating the activity of a hormone, such as a growth hormone, wherein said composition may or may not be administered in conjunction to the administration of said hormone.

Experimental part

Degradation of cytosolic proteins is mainly carried out by the 26S proteasome. The ubiquitin conjugation system selects and targets the proteins for proteasomal degradation by proteolytic cleavage (1). Previously, we have shown that the ubiquitin conjugation system is involved in ligand-induced endocytosis of the growth hormone receptor (GHR)(2). Here, we present direct evidence that proteasome action is required for growth hormone (GH) to be internalized by its receptor. In the presence of specific proteasome inhibitors, GH internalization was inhibited, while the transferrin receptor cycle was unaffected. Consequently, the half-life of the GHR in the presence of ligand was prolonged by proteasome inhibitors. GH uptake by a truncated GHR proceeded normally in the presence of inhibitors. Experiments with CHO cells harboring a temperature-sensitive ubiquitin-activating enzyme (E1) showed that the ubiquitin conjugating system is required before the proteasome can act upon the GHR.

Down regulation of signal transducing membrane receptors is part of highly programmed cascades of events leading both to extinction of the signalling pathway(s) and to rapid degradation of the primary messenger: the receptor and its ligand(3, 4, 5, 6, 7). In the absence of ligand the half-life of GHR is approximately 1-2 h depending on the cell system used. The assumption is that this is mainly due to a proteolytic cleavage in the extracellular domain of the GHR resulting in soluble GH-binding protein(8). If ligand is present, a completely different scenario follows: two GHR polypeptides dimerize, they are phosphorylated by the tyrosine kinase Jak2 and ubiquitinated, and the complex is endocytosed. As the ubiquitin conjugation system acts generally in concert with the 26S proteasome, we examined the

effect of proteasome inhibitors on Cy3-labelled GH uptake. CHO cells carrying a temperature sensitive E1 enzyme and expressing the rabbit GHR were incubated at the permissive temperature with Cy3-GH(9, 10). Incubation for 30 min resulted in abundant fluorescent label in endosomal and lysosomal compartments (Fig. 1A). If the cells were treated with the specific proteasome inhibitors MG132 (Fig. 1D) and lactacystin (Fig. 1G) hardly any label was present intracellularly. The same results were obtained if the cells were treated with carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone or a more membrane permeable analogue of lactacystin, its b-lactone form (not shown)(11, 12). To ascertain that these proteasome inhibitors did not cause pleiotropic effects on the receptor mediated endocytotic machinery we used Cy3-labeled transferrin under identical conditions (Fig. 1C,1F,1I); no inhibition of transferrin uptake was observed. To address the question whether the proteasome acts directly on the GHR we used CHO cells expressing a GHR, truncated after amino acid residue 369 (GHR 1-369, with amino acid residues 370-620 deleted). Fig. 1B, 1E, 1H show that the same inhibitors as used for the full-length GHR had no effect on GH endocytosis by the truncated GHR. Most likely, removal of a portion of the cytosolic tail is sufficient to enable endocytosis of GH.

To confirm and quantify the effect of the proteasome inhibitors we measured the uptake of ^{125}I-GH in a kinetic experiment. Cells were pretreated with the inhibitors, ^{125}I-GH was bound on ice, and cells were incubated for various periods of time (Fig. 2). At the end of the incubation period uptake was determined by washing the cells at low pH to remove label from the cell surface. Again, GH uptake was inhibited by the proteasome inhibitors: MG132 and lactacystin b-lactone reduced the uptake to approximately 25% of the control level. Lactacystin was somewhat less effective,

probably due to its poor cell-permeant properties. TCA-soluble radioactivity in the culture medium, derived from lysosomal degradation of ^{125}I-GH increased after 15 minutes if no inhibitor was added; virtually no TCA-soluble radioactivity appeared if the inhibitors were present (not shown). These results show that receptor mediated uptake and degradation of GH is severely inhibited in the presence of proteasome inhibitors.

If proteasome inhibitors affect GHR-uptake, it is expected that the inhibitors prolong the lifetime of GHRs at the cell surface. To address this, we measured the effect of MG132 in a pulse-chase experiment. Fig. 3A clearly shows that MG132 affects the degradation rate of mature GHR; it does not affect membrane transport from ER to the Golgi complex as is clear from the undisturbed maturation kinetics (i.e. the conversion of 110 kDa species to the mature 130 kDa protein). To assess the effect of MG132 more directly we measured degradation of cell-surface biotinylated GHR after incubation in the presence of ligand (Fig. 3B). As expected, GH induced a rapid disappearance of the GHR. However, clearance from the cell surface was reduced >50% in the presence of MG132. Interestingly, a biotinylated 85-kDa intermediate degradation product visualised with a monoclonal antibody specific for the extracellular domain appeared transiently. The lifetime of this species was extended in the presence of MG132. Attempts to accumulate this species or other degradation products failed until now. In the absence of GH, biotinylated GHRs had a half life of approximately 60-90 min at 30°C, independent of the presence of MG132 as has been shown previously for the steady state situation(4, 5, 6). Constitutive cleavage of cell surface GHR resulted in soluble GH-binding protein in the culture medium (not shown).

To investigate whether the ubiquitin system selects the GHR for truncation by the proteasome we expressed the

receptor in CHO-ts20 cells harboring a temperature-sensitive ubiquitin activating enzyme, E1. The cells were incubated for 60 min at 42°C (sufficient to stop generation of newly ubiquitinated proteins) followed by 3 h at 40°C in the presence or absence of GH, and the amounts of GHR were determined by quantitative western blot analysis (Fig. 4). At the permissive temperature, incubation in the presence of GH decreased the steady state amounts of GHR. If the cells were kept at 40°C in the presence of GH the amount of GHR increased to 170%. To ascertain that this increase is solely due to a defective ubiquitin conjugation system we used the CHO-E36 cells with an intact ubiquitin conjugation system at 40°C. As expected, in these cells the steady state of mature GHR decreased to approximately 50% of that without GH both at 30 and 40°C. The experiment clearly shows that, if the ubiquitin system is switched off, the GHR is stabilized in the presence of GH, rather than degraded as is the case in the (wild-type) CHO-E36 cells. The conclusion is that the ubiquitin conjugation system targets the GHR for partial degradation by the proteasome.

It has been generally accepted that the ubiquitin/proteasome system is involved in selective degradation of cytosolic and nuclear proteins(1). At the cytosolic face of the endoplasmic reticulum the ubiquitin/proteasome system is involved in degradation of mal-folded ER proteins(13, 14). In a growing number of cases the ubiquitin conjugation system seems to be involved in the selecting steps directly preceding endocytosis at the plasma membrane. In yeast the a-factor receptor Ste2p(15), the Ste6 ABC transporter(16), Gap1p amino acid permease(17), Fur4 uracyl permease(18), and Pdr5(19), a multidrug transporter, are all ubiquitin-dependently endocytosed. Previously, we have shown that GHR endocytosis requires an intact ubiquitin system and that GH internalization is accompanied by GHR

ubiquitination(2, 10). Here, we present direct evidence that for the same event proteasome action is required as well. The proteasome cleaves or truncates the tail of the dimerized receptor, as truncation of the GHR at amino acid residue 369 renders the ligand-induced endocytosis of the receptor insensitive to the action of proteasome inhibitors. In addition, the transient appearance of a 85-kDa degradation intermediate again demonstrates a proteasome action on the GHR. Previously, we have shown for truncated GHRs that endocytosis and ubiquitination are closely linked events(2, 10). Together, the data support a model in which specific members of the E2/E3 enzyme families recognize and ubiquitinate the dimerized cytosolic GHR tails; this event induces recruitment of 26S proteasomes, which truncate the tail. Only then the receptor-ligand complex can access the coated pits for further transport to the lysosomes. This model also predicts an early abrogation of part of the signal transduction by the proteasome, while the receptor is still at the cell surface.

Furthermore, we present evidence that the proteasome is also involved in growth hormone receptor downregulation: in the presence of specific proteasomal inhibitors, GH internalization was inhibited, whereas the transferrin receptor cycle remained unaffected. A truncated GH receptor entered the cells independent of proteasome action. Full-length GH receptor disappeared immediately upon endocytosis of ligand. Distal GH receptor tail epitopes became extinct rapidly upon endocytosis; loss of the cytosolic tail was ligand-dependent and preceded degradation of luminal epitopes. Apparently the proteasome truncates a portion of the growth hormone receptor tail before endocytosis can proceed. This implies that signal transduction continues intracellularly with different characteristics compared to GHR signalling at the cell surface.

Although these findings apply to the GHR function, there are indications that the ubiquitin system is involved in regulating the resident time at the cell surface of other membrane proteins. The Met tyrosine kinase receptor degradation is partly dependent on proteasome action(20), the resident time of the sodium channel protein ENac is regulated by the ubiquitin system(21), while many signalling membrane receptors e.g. the TCR z-chain(22), the Kit-(23), EGF-(24), IgE-(25, 26), and prolactin(26) receptor are all ubiquitinated upon activation.

Removal of the extracellular portion of the GHR (the binding domain) occurs continuously (at steady state) if a receptors reside at the cell surface and if no ligand (GH) is bound. Comparison of the molecular sizes of remnant receptor, remaining after proteolytic digestion of cells bearing GHR at their cell surface using an aspecific protease like proteinase K, with the size of the remnant receptor cleaved in vivo by membrane-associated endogenous proteolytic activity demonstrates that the natural cleavage site is very close to the plasma membrane. This is confirmed by the observation that the apparent molecular size of the remnant GHR cleaved with trypsin (cleaving e.g. after lysine residue 221, 25 amino acids upstream) is approximately 3 kDa larger than observed for that of the in vivo generated remnant GHR (Fig. 5). The experiment shows that upon proteinase K treatment the 130 kDa GHR was converted to a 70-kDa species. A similar band was visible if the cells were incubated in the presence of the proteasome inhibitor MG132. If trypsin was used instead of proteinase K a slightly bigger species was observed. If the phenylalanine-327 was converted to an alanine in the receptor tail (which aborts ubiquitination and endocytosis of the receptor) similar results as in wildtype receptor were observed. The conclusion is that the GHR is cut very close to its transmembrane segment by a yet unknown proteolytic enzyme. Combining this finding with the nature of the amino acid sequence 3 kDa downstream from the trypsin

sensitive site we propose that the specificity of the proteolytic cleavage resides at or around the amino acid sequence CEEDFYR.

Methods

Cells and antibodies. A polyclonal antibody to GHR was raised in rabbits against amino acid residues 327-493 as described(10); antibody (Mab5) recognizing the luminal part of the GHR was from AGEN Inc, Parsippany, NJ. A Chinese hamster ovary (CHO) cell line, harboring a temperature-sensitive defect in the ubiquitin activation enzyme E1 (CHO-ts20), was transfected with both the full length rabbit GHR cDNA sequence and a truncated GHR (1-369)(9, 10). 10 mM sodium butyrate was added to the cells 18 h before use to increase GHR expression(2, 27). As control CHO-E36 cells were used stably transfected with GHR.

GH binding and internalization. ^{125}I-hGH was prepared using chloramine T(28). For internalization studies, cells were grown in 35 mm dishes, washed with aMEM, supplemented with 20 mM HEPES, incubated for 1 h at 30°C in aMEM/HEPES, ^{125}I-GH (8 nM) was bound on ice for 60 min in the absence or presence of excess unlabelled GH, and the cells were washed free of unbound GH and incubated for 0 to 60 min. If indicated, lactacystin or its b-lactone (20 mM), MG132 (20 mM), and ZL_3vinylsulfone (20 mM), dissolved in either ethanol or dimethylsulfoxide, or only vehicle were added 3 h before the start of the experiment. Membrane-associated GH was removed by acid wash (0.15 M NaCl, 0.05 M glycine, pH 2.5)(29) and internalized GH was determined by measuring the radioactivity after solubilisation of the acid-treated cells by 1 M NaOH.

Cell surface biotinylation. CHO-ts20 cells, grown in 35 mm dishes, were incubated for 60 min at 30°C in aMEM/HEPES, biotinylated on ice for 30 min using sulfo-NHS-SS-biotin (Pierce, Rockford, IL), and washed free of biotinylating

reagents. For GH binding, the cells were incubated for 1 h on ice in the presence of 16 nM GH with either 20 mM MG132 or 1% ethanol. The incubation was continued at 30°C for various time periods in the presence of GH with MG132 or ethanol only. Cells were then lysed on ice, and equal aliquots of the cells extracts were incubated with streptavidin beads for 1 h at 4°C, and washed with PBS.

Metabolic labelling. The cells were incubated in methionine-free MEM for 30 min and then ^{35}S-methionine (TRAN-^{35}S label, 3.7 MBq/ml, 40 TBq/mmol, ICN, CA, USA) was added and the incubation was continued for 30 min; the radioactivity was chased in the presence of 0.1 mM of unlabelled methionine. Cell lysates were subjected to immunoprecipitation. The radioactivity was determined using a Molecular Dynamics phosphoimager (STORM860 equipped with ImageQuant software, Molecular Dynamics, Sunnyvale, CA).

Cell lysis, immunoprecipitation and western blotting. At the end of the incubation, cells were immediately washed and lysed on ice in 0.3 ml of 1% Triton X-100, 1 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM MG132, and 1 mM phenylmethylsulphonyl fluoride in PBS. Equal aliquots of the cell extracts were subjected to SDS polyacrylamide gel electrophoresis and immunoblotting as described(2). For detection we used the enhanced chemifluorescence system (Amersham Corp, UK).

Microscopy. Cy3-GH and Cy3-transferrin were prepared using a Fluorolink-Cy3 label kit according to the supplier's instructions (Amersham, UK). The cells, grown on coverslips, were incubated for 60 min in aMEM, supplemented with 20 mM HEPES at 30°C and for 30 min with Cy3-GH (1 mg/ml) or Cy3-transferrin (20 mg/ml). Cells were washed with PBS to remove unbound label and fixed for two hours in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol and confocal laser scanning microscopy was performed using a

Leica TCS 4D system.

Extra -cellular proteolytic removal or truncation of growth hormone binding protein. Cells (CHO cells expressing GHR-wt or GHR F327A) were labelled in the presence of [35S]methionine for 4 h in the presence or absence of the proteasome inhibitor MG132 (20μM)[10]. The cells were either treated with proteinase K (0.5mg/ml) or with trypsin (0.5mg/ml) on ice for 30 min or not treated. The proteases were removed, the cells were lysed, and the radioactively labelled GHR was immunoprecipitated using an anti-cytosolic tail antibody, analysed by SDS-PAGE, and visualised by phosphoimaging.

Legends to the figures

Fig. 1. *Effect of proteasome inhibitors on Cy3-GH and Cy3-transferrin endocytosis.*
CHO-ts20 cells, expressing either wild type (A,D,G,C,F,I), or truncated GHR (B,E,H) were incubated with vehicle (A-C) or with 20 mM MG132 (D-F) or 20 mM lactacystin (G-I) for 3 h at 30°C; then Cy3-GH (A,B,D,E,G,H), or Cy3-transferrin (C,F,I) were added for 30 min, and the cells were washed, fixed and the fluorescence was visualized by confocal microscopy. If excess unlabelled ligand was added no uptake was observed. If the cells in D,G were treated at pH 2.5 before fixation, virtually no label was visible (not shown).

Fig. 2. *Proteasome inhibitors inhibit uptake of ^{125}I-GH.*
CHO-ts20 cells were incubated with or without inhibitors for 3 h at 30°C, and put on ice for 60 min with ^{125}I-GH. The cells were then incubated at 30°C as indicated. Background label was determined in untransfected cells and subtracted. Plotted are the amounts of ^{125}I-GH internalized as a percentage of the cell-associated radioactivity at the start of incubation.
□, control (1% ethanol); ●, MG132; O, lactocystin; X, lactacystin β-lactone.

Fig. 3. *Effect of MG132 on GHR degradation in the presence of ligand.* A. Cells were pulse-labelled with ^{35}S-methionine and then chased in unlabelled methionine in the presence of 16 nM GH with or without MG132 for the times indicated. GHR was immunoprecipitated using an anti-cytosolic tail antibody. The radioactivity in the mature (upper) band (inset) was quantitated and plotted. □, control (1% ethanol); ●, MG132. B. Cells were biotinylated and incubated in the presence of GH with MG132 or with vehicle only at 30°C for the time

periods indicated. After streptavidin-bead purification aliquots of the cell lysates were analysed by SDS-PAGE; western blots were incubated with a monoclonal antibody specific for the extracellular GHR domain and detected with enhanced chemifluorescence. The sharp band below the mature GHR (130 kDa) originated from a slight background staining of precursor GHR (110 kDa), due to incomplete removal of biotinylation reagent at cell lysis.

Fig. 4. *GH-induced receptor degradation depends on an intact ubiquitin conjugation system.*
CHO-ts20 and CHO-E36 cells were incubated at the permissive or non-permissive temperature for 3 h in the presence or absence of GH. Cells were lysed and aliquots were analysed by western blotting and enhanced chemifluorescence. M, mature GHR, P, precursor GHR. The lower panel shows the quantification (expressed as percentage of mature GHR present without GH).

Fig. 5. *Proteolytic removal of growth hormone binding protein.*
Cells (CHO cells expressing GHR-wt or GHR F327A) were labelled in the presence of [35S]methionine for 4 h in the presence or absence of the proteasome inhibitor MG132 (20μM)[10]. The cells were either treated with proteinase K (0.5mg/ml) or with trypsin (0.5mg/ml) on ice for 30 min or not treated. The proteases were removed, the cells were lysed, and the radioactively labelled GHR was immunoprecipitated using an anti-cytosolic tail antibody, analysed by SDS-PAGE, and visualised by phosphoimaging.

Table 1

SWVEFIELDI	GHR sequence (human, rabbit.....)
LWVEFIELDI	GHR chicken
LLVEYLEVDD	prolactin receptor, human
LLVEFLENDD	prolactin receptor, rabbit, rat, mouse
DNVDYLTRDW	Ca^{++}-channel vertebrate skeletal muscle
QAAEYLRSET	TKR CEK_2, PIG (FGF-receptor family)
IDAEYISAER	Transmembrane receptor sex precursor
LKGEFIWVDG	IgE receptor
YGSEYINLDG	angiotensin converting enzyme
SEGEYIPLDQ	potassium channel IRK_1
DGHEYIYVDP	PDGF receptor α-chain
DGHEYIYVDP	PDGF receptor β-chain
DNFEYLTRDS	Ca^{++}-channel α1β (human, rat, rabbit)
KIFEYLRRDT	Cl^--channel, CLC7
SLQEYLQNDT	Tyrosine-protein kinase FRK (human)
TELEYLGPDE	Glut4 Ins-regulated glucose transporter
NQEEYLRYDS	MHC-IIβ (rat)
ENPEYLGLDV	ERB2 TKR (neu-oncogene)
RLKEYLAGDV	Anion transporter I
LYKDFLTLEH	Vascular endothelial growth factor receptor 2
EQLEYLSYDA	Vascular endothelial growth factor receptor 3
PEGEFLPLDQ	G protein-act. inward rectifier K^+-channel-1
SDSEFLLPDT	Protein-tyrosine phosphatase zeta (human)
SALDFIRRES	Glutamate (NMDA) receptor subunit epsilon 2
AHNEYLVSEI	Rhesus blood group-associated glycoprotein
VTLDFLDAEL	Dihydropyridine-sensitive l-type, Ca^{++}-channel
EISDFLRYEL	Thrombopoietin receptor
SAKDYIYQDS	Serotonin receptor 1B (brain)
YQQDFFPKEA	Epidermal growth factor receptor
SKLQYILAQI	Sodium-, chloride-dependent transporter NTT4
TPLNYILLNL	Rhodopsin
TSVDLLDINV	Interleukin-2 receptor β-chain
GTPDYIAPEI}	cAMP-dependent protein kinase C, alpha, beta,
GTPEYLAPEI}	delta, epsilon, gamma
LVFEYLDKDL	Serine/threonine kinase PCTAIRE 1,2
LVFEYLDSDL	Serine/threonine kinase PCTAIRE 3
IGADFLTKEV	Small GTP-binding protein Rab-7
IGVEFLNKDL	Small GTP-binding protein Rab-9
ISVEFLVLDS	Synaptotagmin IV
SDIDFLIEEI	Glutamate decarboxylase (GAD67)

AIGEFILVDK	Fructose 1,6 diphosphatase (FBPase)
QKQEYKTLEY	Cystic fibrosis transmembrane conductance regulator (CFTR)
PPPxY->WWdomain of NEDD4	Epithelial Na^+-channel (ENaC)

REFERENCES

1. A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* in press (1998).

2. R. Govers, P. van Kerkhof, A. L. Schwartz, G. J. Strous, *Embo J* 16, 4851-8 (1997).

3. L. S. Argetsinger, C. CarterSu, in *Physiol Rev* . (1996), vol. 76,, pp. 1089-1107.

4. P. Roupas, A. Herington, *Mol. Cell. Endocrinol.* 57, 93-99. (1988).

5. E. G. Gorin, H.M., *Endocrinology* 116, 1796-1805. (1985).

6. M. A. Lesniak, J. Roth, *J. Biol. Chem.* 251, 372-3729. (1976).

7. R. H. Hackett, et al., *J. Biol. Chem.* 272, 11128-11132. (1997).

8. G. Baumann, M. W. Stolar, K. Amburn, C. P. Barasano, B. C. deVries, *J. Clin.Endocrinol.Metabol.* 62, 134-141. (1986).

9. R. G. Kulka, et al., *J.Biol.Chem.* 263, 15726-15731. (1988).

10. G. J. Strous, P. van Kerkhof, R. Govers, A. Ciechanover, A. L. Schwartz, *Embo J* 15, 3806-12 (1996).

11. M. Bogyo, et al., *Proc. Natl. Acad. Sci. USA* 94, 6619-6634. (1997.).

12. A. Craiu, et al., *J. Biol. Chem.* 272, 13437-13445. (1997).

13. E. J. H. J. Wiertz, et al., *Nature* 384, 432-438. (1996).

14. M. M. Hiller, A. Finger, M. Schweiger, D. H. Wolf, *Science* 273, 1725-1728. (1996).

15. L. Hicke, H. Riezman, *Cell* 84, 277-287. (1996).

16. R. Kolling, C. P. Hollenberg, *EMBO J.* 13, 3261-3271. (1994).

17. C. Hein, B. Andre, *Mol Microbiol* 24, 607-616. (1997).

18. J. M. Galan, V. Moreau, B. Andre, C. Volland, R.

HaguenauerTsapis, *J. Biol. Chem.* 271, 10946-10952. (1996).

19. R. Egner, K. Kuchler, *FEBS Lett.* 378, 177-181. (1996).

20. M. Jeffers, G. A. Taylor, K. M. Weidner, S. Omura, G. F. Vandewoude, *Mol Cell Biol.* 17,, 799-808. (1997).

21. O. Staub, et al., *EMBO J.* 21, 6325-6336. (1997.).

22. D. Hou, C. Cenciarelli, J. P. Jensen, H. B. Nguyen, A. M. Weissman, *J. Biol. Chem.* 269, 14244-14247. (1994).

23. K. Miyazawa, K. Toyama, A. Gotoh, Hendrie, P.C., C. Mantel, H. E. Broxmeyer, *Blood* 83, 137-145. (1994).

24. Z. Galcheva-Gargova, S. J. Theroux, R. J. Davis, in *Oncogene* . (1995), vol. 11,, pp. 2649-2655.

25. R. Paolini, J. P. Kinet, *EMBO J.* 12, 779-786. (1993).

26. C. Cahoreau, L. Garnier, J. Djiane, G. Devauchelle, M. Cerutti, *FEBS Lett.* 350, 230-234. (1994).

27. C. M. Gorman, B. H. Howard, *Nucleic Acids Res.* 11, 7631-7648. (1983).

28. L. J. Murphy, L. Lazarus, *Endocrinology* 115, 1625-1632. (1984).

29. P. Roupas, A. C. Herington, *Mol. Cell Endocrinol.* 47, 81-90. (1986).

SEQUENCE LISTING

<110> Universiteit Utrecht
Strous, Gerardus
Van Kerkhof, Petrus
Govers, Roland

<120> CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIBITORS OR RECEPTOR FRAGMENTS

<130> 2183-4525US

<140> Filed concurrently with application
<141> 2000-09-12

<150> PCT/NL99/00136
<151> 1999-03-12

<150> EP98200799.9
<151> 1998-03-12

<160> 50

<170> PatentIn version 3.0

<210> 1
<211> 8
<212> PRT
<213> Unknown organism

<220>
<221> X
<222> (1)..(8)
<223> Residues 1, 5-6 and 8 can be any amino acid

<220>
<221> E
<222> (2)..(2)
<223> The amino acid E (glutamic acid can be replaced by D

<220>
<221> F
<222> (3)..(3)
<223> The amino acid F can be replaced by Y

<220>
<221> I
<222> (4)..(4)

<223> The amino acid I can be replaced by L, V or F

<220>
<221> D
<222> (8)..(8)
<223> The amino acid D can be replaced by E

<400> 1

Xaa Glu Phe Ile Xaa Xaa Asp Xaa
1 5

<210> 2
<211> 12
<212> PRT
<213> Unknown Organism

<400> 2

Asp Asp Ser Trp Val Glu Phe Ile Glu Leu Asp Ile
1 5 10

<210> 3
<211> 10
<212> PRT
<213> Unknown Organism

<400> 3

Asp Ser Trp Val Glu Phe Ile Glu Leu Asp
1 5 10

<210> 4
<211> 129
<212> PRT
<213> Unknown organism

<400> 4

Ser Lys Gln Gln Arg Ile Lys Met Leu Ile Leu Pro Pro Val Pro Val
1 5 10 15

Pro Lys Ile Lys Gly Ile Asp Pro Asp Leu Leu Lys Glu Gly Lys Leu
20 25 30

Glu Glu Val Asn Thr Ile Leu Ala Ile His Asp Ser Tyr Lys Pro Glu
35 40 45

Phe His Ser Asp Asp Ser Trp Val Glu Phe Ile Glu Leu Asp Ile Asp

50 55 60

Glu Pro Asp Glu Lys Thr Glu Glu Ser Asp Thr Asp Leu Leu Ser Ser
65 70 75 80

Asp His Glu Lys Ser His Ser Asn Leu Gly Val Lys Asp Gly Asp Ser
85 90 95

Gly Arg Thr Ser Cys Cys Glu Pro Asp Ile Leu Glu Thr Asp Phe Asn
100 105 110

Ala Asn Asp Ile His Glu Gly Thr Ser Glu Val Ala Gln Pro Gln Arg
115 120 125

Leu

<210> 5
<211> 38
<212> PRT
<213> Unknown organism

<400> 5

Lys Asp Gly Asp Ser Gly Arg Thr Ser Cys Cys Glu Pro Asp Ile Leu
1 5 10 15

Glu Thr Asp Phe Asn Ala Asn Phe Ile His Glu Gly Thr Ser Glu Val
20 25 30

Ala Gln Pro Gln Arg Leu
35

<210> 6
<211> 10
<212> PRT
<213> Unknown organism

<400> 6

Thr Glu Leu Glu Tyr Leu Gly Pro Asp Glu
1 5 10

<210> 7
<211> 7
<212> PRT
<213> Unknown organsim

<400> 7

Cys Glu Glu Asp Phe Tyr Arg

1 5

<210> 8
<211> 10
<212> PRT
<213> GHR sequence (human, rabbit)

<400> 8

Ser Trp Val Glu Phe Ile Glu Leu Asp Ile
1 5 10

<210> 9
<211> 10
<212> PRT
<213> GHR chicken

<400> 9

Leu Trp Val Glu Phe Ile Glu Leu Asp Ile
1 5 10

<210> 10
<211> 10
<212> PRT
<213> prolactin receptor, human

<400> 10

Leu Leu Val Glu Tyr Leu Glu Val Asp Asp
1 5 10

<210> 11
<211> 10
<212> PRT
<213> prolactin receptor, rabbit, rat, mouse

<400> 11

Leu Leu Val Glu Phe Leu Glu Asn Asp Asp
1 5 10

<210> 12
<211> 10
<212> PRT
<213> Ca++ channel

<400> 12

Asp Asn Val Asp Tyr Leu Thr Arg Asp Trp
1 5 10

<210> 13
<211> 10
<212> PRT
<213> FGF Receptor Family

<400> 13

Gln Ala Ala Glu Tyr Leu Arg Ser Glu Thr
1   5   10

<210> 14
<211> 10
<212> PRT
<213> Transmembrane receptor sex precursor

<400> 14

Ile Asp Ala Glu Tyr Ile Ser Ala Glu Arg
1   5   10

<210> 15
<211> 10
<212> PRT
<213> IgE Receptor

<400> 15

Leu Lys Gly Glu Phe Ile Trp Val Asp Gly
1   5   10

<210> 16
<211> 10
<212> PRT
<213> ANGIOTENSIN CONVERTING ENZYME

<400> 16

Tyr Gly Ser Glu Tyr Ile Asn Leu Asp Gly
1   5   10

<210> 17
<211> 10
<212> PRT
<213> POTASSIUM CHANNEL IRK

<400> 17

Ser Glu Gly Glu Tyr Ile Pro Leu Asp Gln
1   5   10

<210> 18
<211> 10
<212> PRT
<213> PDGF RECEPTOR ALPHA-CHAIN

<400> 18

Asp Gly His Glu Tyr Ile Tyr Val Asp Pro
1 5 10

<210> 19
<211> 10
<212> PRT
<213> PDGF RECEPTOR BETA-CHAIN

<400> 19

Asp Gly His Glu Tyr Ile Tyr Val Asp Pro
1 5 10

<210> 20
<211> 10
<212> PRT
<213> Ca++ -channel

<400> 20

Asp Asn Phe Glu Tyr Leu Thr Arg Asp Ser
1 5 10

<210> 21
<211> 10
<212> PRT
<213> Cl- CHANNEL CLC7

<400> 21

Lys Ile Phe Glu Tyr Leu Arg Arg Asp Thr
1 5 10

<210> 22
<211> 10
<212> PRT
<213> TYROSINE-PROTEIN KINASE FRK

<400> 22

Ser Leu Gln Glu Tyr Leu Gln Asn Asp Thr
1 5 10

<210> 23

<211> 10
<212> PRT
<213> GLUT4 INS-REGULATED GLUCOSE TRANSPORTER

<400> 23

Thr Glu Leu Glu Tyr Leu Gly Pro Asp Glu
1 5 10

<210> 24
<211> 10
<212> PRT
<213> MHC-II(BETA) (RAT)

<400> 24

Asn Gln Glu Glu Tyr Leu Arg Tyr Asp Ser
1 5 10

<210> 25
<211> 10
<212> PRT
<213> ERB2 TKR

<400> 25

Glu Asn Pro Glu Tyr Leu Gly Leu Asp Val
1 5 10

<210> 26
<211> 10
<212> PRT
<213> ANION TRANSPORTER I

<400> 26

Arg Leu Lys Glu Tyr Leu Ala Gly Asp Val
1 5 10

<210> 27
<211> 10
<212> PRT
<213> VASCULAR ENDOTHELIAL GROWTH FACTOR

<400> 27

Leu Tyr Lys Asp Phe Leu Thr Leu Glu His
1 5 10

<210> 28
<211> 10

<212> PRT
<213> VASCULAR ENDOTHELIAL GROWTH FACTOR

<400> 28

Glu Gln Lys Glu Tyr Lys Ser Tyr Asp Ala
1   5   10

<210> 29
<211> 10
<212> PRT
<213> G PROTEIN-ACT. INWARD RECTIFIER K+-CHANNEL-1

<400> 29

Pro Glu Gly Glu Phe Leu Pro Leu Asp Gln
1   5   10

<210> 30
<211> 10
<212> PRT
<213> PROTEIN-TYROSINE PHOSPHATASE ZETA

<400> 30

Ser Asp Ser Glu Phe Leu Leu Pro Asp Thr
1   5   10

<210> 31
<211> 10
<212> PRT
<213> GLUTAMATE (NMDA) RECEPTOR SUBUNIT EPSILON 2

<400> 31

Ser Ala Leu Asp Phe Ile Arg Arg Glu Ser
1   5   10

<210> 32
<211> 10
<212> PRT
<213> RHESUS BLOOD GROUP-ASSOCIATED GLYCOPROTEIN

<400> 32

Ala His Asn Glu Tyr Leu Val Ser Glu Ile
1   5   10

<210> 33
<211> 10
<212> PRT

<213> DIHYDROPYRIDINE-SENSITIVE 1-TYPE

<400> 33

Val Thr Leu Asp Phe Leu Asp Ala Glu Leu
1 5 10

<210> 34
<211> 10
<212> PRT
<213> THROMBOPOIETIN RECEPTOR

<400> 34

Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu
1 5 10

<210> 35
<211> 10
<212> PRT
<213> SEROTONIN RECEPTOR 1B

<400> 35

Ser Ala Lys Asp Tyr Ile Tyr Gln Asp Ser
1 5 10

<210> 36
<211> 10
<212> PRT
<213> EPIDERMAL GROWTH FACTOR

<400> 36

Tyr Gln Gln Asp Phe Phe Pro Lys Glu Ala
1 5 10

<210> 37
<211> 10
<212> PRT
<213> SODIUM, CHLORIDE-DEPENDENT TRANSPORTER NTT4

<400> 37

Ser Lys Leu Gln Tyr Ile Leu Ala Gln Ile
1 5 10

<210> 38
<211> 10
<212> PRT
<213> RHODOPSIN

<400> 38

Thr Pro Leu Asn Tyr Ile Leu Leu Asn Leu
1 5 10

<210> 39
<211> 10
<212> PRT
<213> INTERLEUKIN-2 RECEPTOR BETA-CHAIN

<400> 39

Thr Ser Val Asp Leu Leu Asp Ile Asn Val
1 5 10

<210> 40
<211> 10
<212> PRT
<213> cAMP-DEPENDENT PROTEIN KINASE C, ALPHA, BETA

<400> 40

Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile
1 5 10

<210> 41
<211> 10
<212> PRT
<213> cAMP-DEPENDENT PROTEIN KINASE DELTA, EPSILON, GAMMA

<400> 41

Gly Thr Pro Glu Tyr Leu Ala Pro Glu Ile
1 5 10

<210> 42
<211> 10
<212> PRT
<213> SERINE/THREONINE KINASE PCTAIRE 1,2

<400> 42

Leu Val Phe Glu Tyr Leu Asp Lys Asp Leu
1 5 10

<210> 43
<211> 10
<212> PRT
<213> SERINE/THREONINE KINASE PCTAIRE 3

<400> 43

Leu Val Phe Glu Tyr Leu Asp Ser Asp Leu
1   5   10

<210> 44
<211> 10
<212> PRT
<213> SMALL GTP-BINDING PROTEIN Rab-7

<400> 44

Ile Gly Ala Asp Phe Leu Thr Lys Glu Val
1   5   10

<210> 45
<211> 10
<212> PRT
<213> SMALL GTP-BINDING PROTEIN Rab-9

<400> 45

Ile Gly Val Glu Phe Leu Asn Lys Asp Leu
1   5   10

<210> 46
<211> 10
<212> PRT
<213> SYNAPTOTAGMIN IV

<400> 46

Ile Ser Val Glu Phe Leu Val Leu Asp Ser
1   5   10

<210> 47
<211> 10
<212> PRT
<213> GLUTAMATE DECARBOXYLASE (GAD67)

<400> 47

Ser Asp Ile Asp Phe Leu Ile Glu Glu Ile
1   5   10

<210> 48
<211> 10
<212> PRT
<213> FRUCTOSE 1,6 DIPHOSPHATASE (FBPase)

<400> 48

Ala Ile Gly Glu Phe Ile Leu Val Asp Lys
1   5   10

<210> 49
<211> 10
<212> PRT
<213> CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

<400> 49

Gln Lys Gln Glu Tyr Lys Thr Leu Glu Tyr
1   5   10

<210> 50
<211> 5
<212> PRT
<213> EPITHELIAL Na+ CHANNEL

<220>
<221> X
<222> (4)..(4)
<223> X can be any amino acid

<400> 50

Pro Pro Pro Xaa Tyr
1   5

CLAIMS

1. A method for controlling or up-regulating the availability or activity of a protein comprising regulating binding of the ubiquitin/proteasome system at a ubiquitin/proteasome system binding site of said protein.

2. A method according to claim 1 wherein said binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto, wherein D is the amino acid aspartic acid, E is the amino acid glutamic acid, F is the amino acid phenylalanine, I is the amino acid isoleucine and X is any other amino acid.

3. A method for controlling the availability and/or signal transduction capability of a cell surface receptor comprising providing an inhibitor capable of inhibiting proteolytic cleavage of said receptor.

4. A method according to claim 3 wherein said inhibitor is capable of inhibiting proteolytic cleavage of an intra-cellular part of said receptor.

5. A method according to claim 3 wherein said inhibitor is capable of inhibiting proteolytic cleavage of an extra-cellular part of said receptor.

6. A method according to anyone of claims 3 to 5 wherein said receptor is a hormone receptor, preferably selected from a group consisting of amino acid derivative, prostaglandine, peptide or protein hormone receptors.

7. A method according to claim 6 wherein said receptor is a growth hormone receptor.

8. A method according to any one of claims 1 or 2 wherein said protein is a transport protein.

9. A method according to claim 8 wherein said transport protein is Glut4 insulin regulated glucose transporter.

10. A (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid

sequence located at or around a ubiquitin/proteasome system binding site located in a protein.

11. A (poly)peptide or (poly)peptide analogue or mimeticum according to claim 10 wherein said binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto, wherein D is the amino acid aspartic acid, E is the amino acid glutamic acid, F is the amino acid phenylalanine, I is the amino acid isoleucine and X is any other amino acid.

12. An inhibitor capable of inhibiting proteolytic cleavage of a cell surface receptor for use in a method according to anyone of claims 1 to 7.

13. An inhibitor according to claim 12 which is capable of inhibiting proteolytic cleavage of the intra-cellular part of said receptor.

14. An inhibitor according to claim 13 selected from the group of proteasome inhibitors, such as MG132, carboxybenzyl-leucyl-leucyl-leucinal, lactacystin, carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone or the β-lacton form of lactacystin.

15. An inhibitor according to claim 13 comprising a (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a ubiquitin and/or ubiquitin/proteasome system binding site located in the intra-cellular part of a cell-surface receptor.

16. An inhibitor according to claim 15 wherein said binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto, wherein D is the amino acid aspartic acid, E is the amino acid glutamic acid, F is the amino acid phenylalanine, I is the amino acid isoleucine and X is any other amino acid.

17. An inhibitor according to claim 16 wherein said binding site comprises the amino acid sequence DDSWVEFIELDI or DSWVEFIELD.

18. An inhibitor according to claim 12 capable of inhibiting proteolytic cleavage of a extra-cellular part of said receptor.

19. An inhibitor according to claim 18 wherein said extra-cellular part comprises a approximately 60 kDa fragment of a extra-cellular domain of the growth hormone receptor.

20. An inhibitor according to claim 18 or 19 comprising a (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a proteolytic cleavage signal site located in a extra-cellular part of said receptor.

21. An inhibitor according to claim 20 wherein said cleavage signal site comprises the amino acid sequence CEEDFYR or a sequence essentially corresponding thereto.

22. A (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a ubiquitin and/or ubiquitin/proteasome system binding site located in the intra-cellular part of a cell-surface receptor.

23. A (poly)peptide or (poly)peptide analogue or mimeticum according to claim 22 wherein said binding site comprises the amino acid sequence motif xEFIxxDx or a sequence essentially corresponding thereto, wherein D is the amino acid aspartic acid, E is the amino acid glutamic acid, F is the amino acid phenylalanine, I is the amino acid isoleucine and X is any other amino acid.

24. A (poly)peptide or (poly)peptide analogue or mimeticum that is derived from, competes with or binds to an amino acid sequence located at or around a proteolytic cleavage signal site located in the extra-cellular part of a receptor.

25. A (poly)peptide or (poly)peptide analogue or mimeticum according to claim 24 wherein said cleavage signal site comprises the amino acid sequence CEEDFYR or a sequence essentially corresponding thereto.

26. A pharmaceutical composition comprising an inhibitor according to any of claims 12-21 or a (poly)peptide or

(poly)peptide analogue or mimeticum according to any of claims 10, 11 or 22-25.

27. A pharmaceutical composition according to claim 26 for regulating the activity of a hormone.

28. A pharmaceutical composition according to claim 27 for administering in conjunction with a hormone.

29. Use of an inhibitor according to any of claims 12 to 21 or a (poly)peptide or (poly)peptide analogue or mimeticum according to any of claims 10, 11 or 22-25 for the production of a pharmaceutical composition for controlling the availability and/or signal transduction capability of a cell surface receptor.

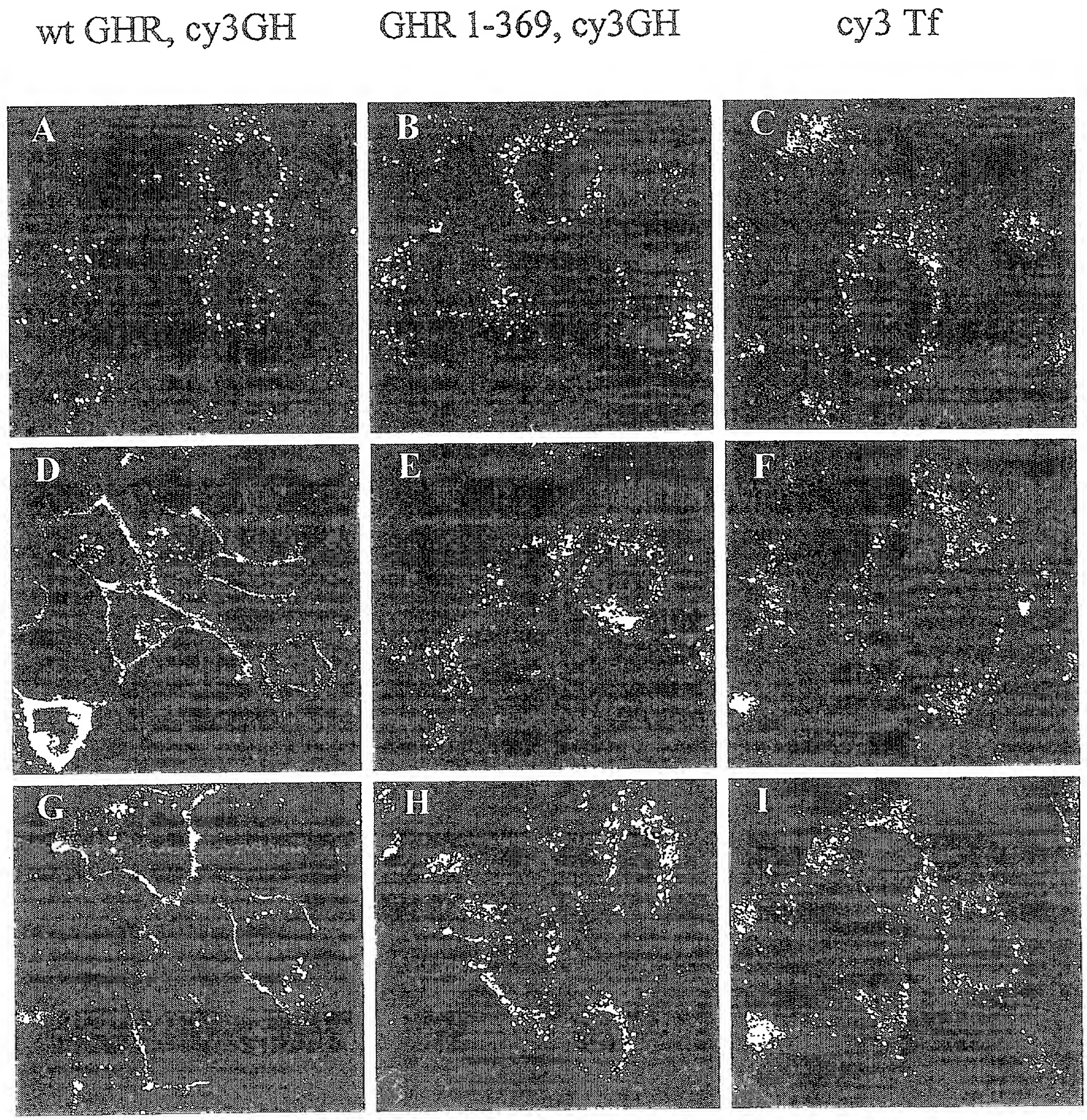
30. Use according to claim 29 for the production of a pharmaceutical composition regulating the activity of a hormone.

31. Use according to claim 29 or 30 wherein said composition is administered in conjunction to the administration of said hormone.

32. Use according to any of claims 29 to 30 for the production of a pharmaceutical composition for the treatment of muscle wasting.

33. A method to control or up-regulate hormone activity by using an inhibitor according to any of claims 12 to 21 or a pharmaceutical composition according to any of claims 26 to 28.

Fig. 1



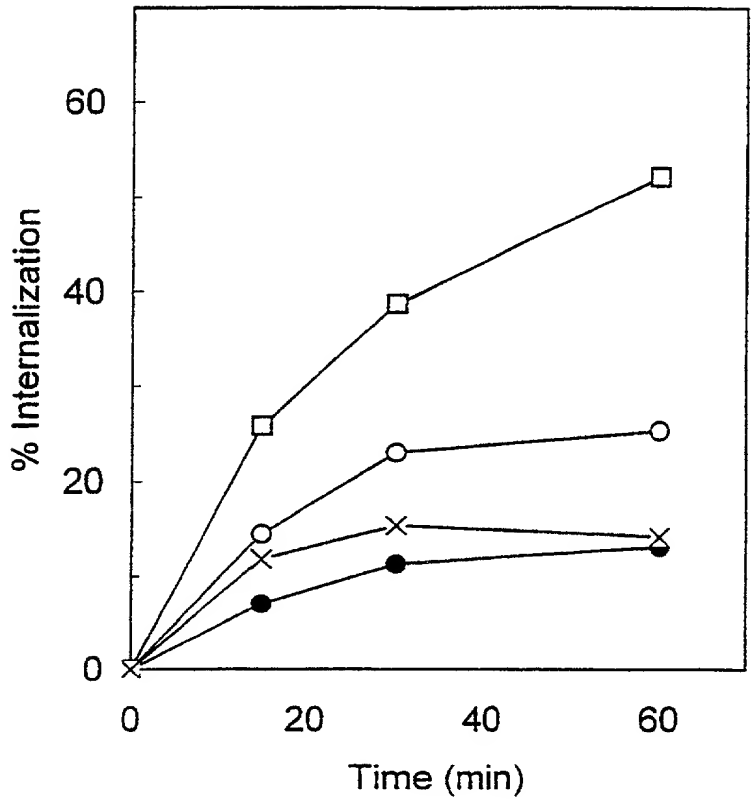


Fig. 2

Fig. 3

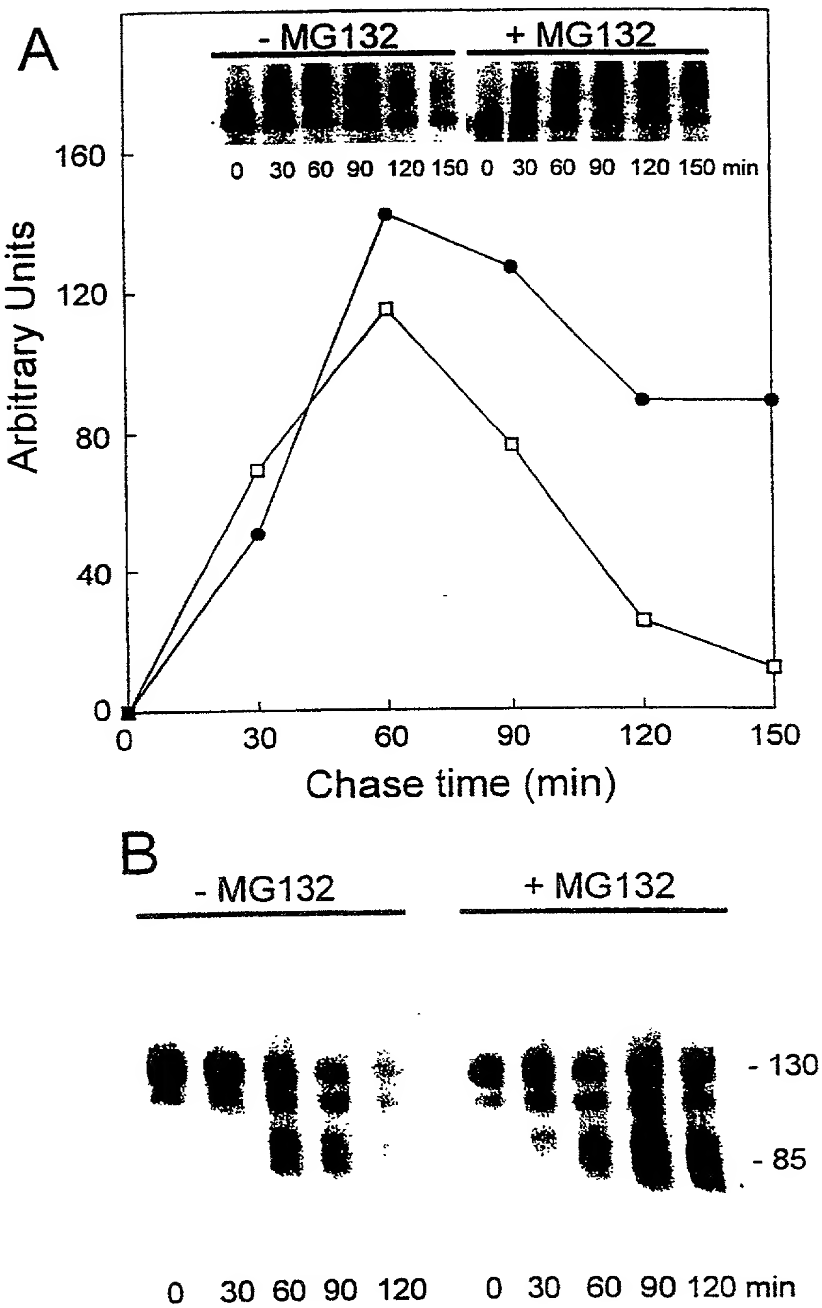
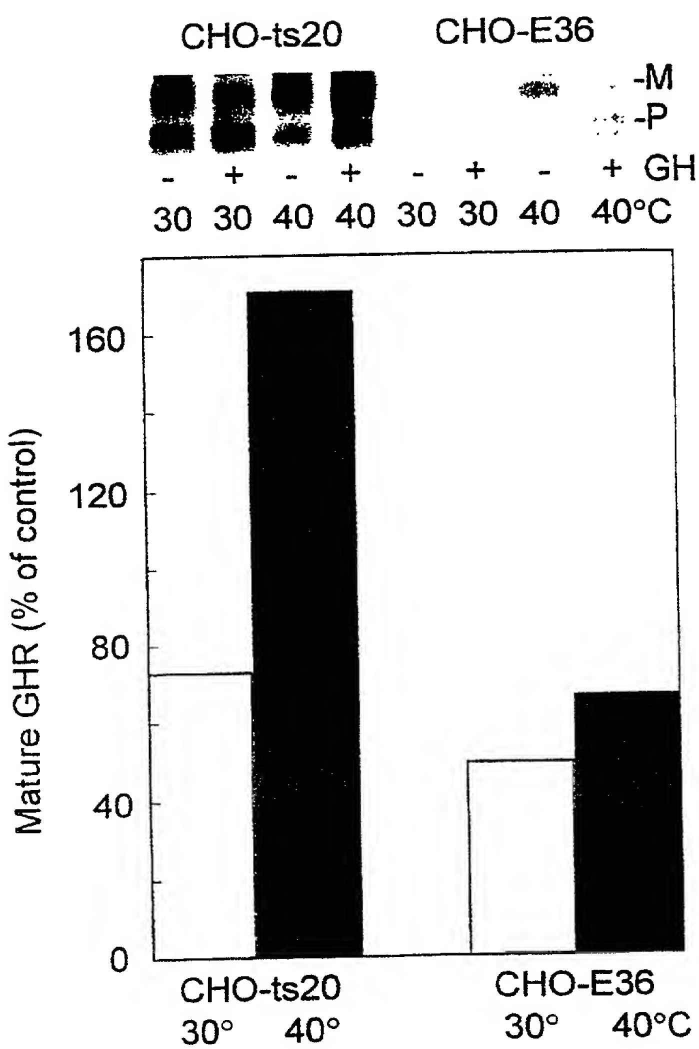

A
- MG132
+ MG132
0 30 60 90 120 150 0 30 60 90 120 150 min
160
120
80
40
0
Arbitrary Units
0 30 60 90 120 150
Chase time (min)
B
- MG132
+ MG132
- 130
- 85
0 30 60 90 120 0 30 60 90 120 min

Fig. 4


CHO-ts20
CHO-E36
-M
-P
- + - + - + - + GH
30 30 40 40 30 30 40 40°C
Mature GHR (% of control)
0
40
80
120
160
CHO-ts20
30° 40°
CHO-E36
30° 40°C

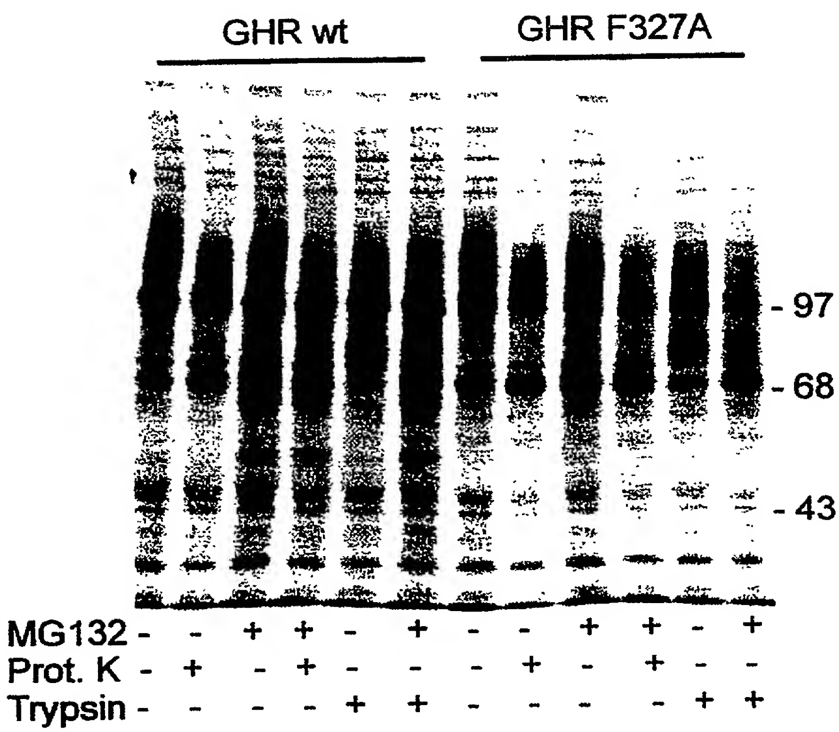


Fig. 5

SEQUENCE LISTING

<110> Universiteit Utrecht
Strous, Gerardus
Van Kerkhof, Petrus
Govers, Roland

<120> CONTROLLING AVAILABILITY OR ACTIVITY OF PROTEINS BY USE OF PROTEASE INHIBITORS OR RECEPTOR FRAGMENTS

<130> 2183-4525US

<140> Filed concurrently with application
<141> 2000-09-12

<150> PCT/NL99/00136
<151> 1999-03-12

<150> EP98200799.9
<151> 1998-03-12

<160> 50

<170> PatentIn version 3.0

<210> 1
<211> 8
<212> PRT
<213> Unknown organism

<220>
<221> X
<222> (1)..(8)
<223> Residues 1, 5-6 and 8 can be any amino acid

<220>
<221> E
<222> (2)..(2)
<223> The amino acid E (glutamic acid can be replaced by D

<220>
<221> F
<222> (3)..(3)
<223> The amino acid F can be replaced by Y

<220>
<221> I
<222> (4)..(4)

<223> The amino acid I can be replaced by L, V or F

<220>
<221> D
<222> (8)..(8)
<223> The amino acid D can be replaced by E

<400> 1

Xaa Glu Phe Ile Xaa Xaa Asp Xaa
1 5

<210> 2
<211> 12
<212> PRT
<213> Unknown Organism

<400> 2

Asp Asp Ser Trp Val Glu Phe Ile Glu Leu Asp Ile
1 5 10

<210> 3
<211> 10
<212> PRT
<213> Unknown Organism

<400> 3

Asp Ser Trp Val Glu Phe Ile Glu Leu Asp
1 5 10

<210> 4
<211> 129
<212> PRT
<213> Unknown organism

<400> 4

Ser Lys Gln Gln Arg Ile Lys Met Leu Ile Leu Pro Pro Val Pro Val
1 5 10 15

Pro Lys Ile Lys Gly Ile Asp Pro Asp Leu Leu Lys Glu Gly Lys Leu
20 25 30

Glu Glu Val Asn Thr Ile Leu Ala Ile His Asp Ser Tyr Lys Pro Glu
35 40 45

Phe His Ser Asp Asp Ser Trp Val Glu Phe Ile Glu Leu Asp Ile Asp

50 55 60

Glu Pro Asp Glu Lys Thr Glu Glu Ser Asp Thr Asp Leu Leu Ser Ser
65 70 75 80

Asp His Glu Lys Ser His Ser Asn Leu Gly Val Lys Asp Gly Asp Ser
85 90 95

Gly Arg Thr Ser Cys Cys Glu Pro Asp Ile Leu Glu Thr Asp Phe Asn
100 105 110

Ala Asn Asp Ile His Glu Gly Thr Ser Glu Val Ala Gln Pro Gln Arg
115 120 125

Leu

<210> 5
<211> 38
<212> PRT
<213> Unknown organism

<400> 5

Lys Asp Gly Asp Ser Gly Arg Thr Ser Cys Cys Glu Pro Asp Ile Leu
1 5 10 15

Glu Thr Asp Phe Asn Ala Asn Phe Ile His Glu Gly Thr Ser Glu Val
20 25 30

Ala Gln Pro Gln Arg Leu
35

<210> 6
<211> 10
<212> PRT
<213> Unknown organism

<400> 6

Thr Glu Leu Glu Tyr Leu Gly Pro Asp Glu
1 5 10

<210> 7
<211> 7
<212> PRT
<213> Unknown organsim

<400> 7

Cys Glu Glu Asp Phe Tyr Arg

1   5

<210> 8
<211> 10
<212> PRT
<213> GHR sequence (human, rabbit)

<400> 8

Ser Trp Val Glu Phe Ile Glu Leu Asp Ile
1   5   10

<210> 9
<211> 10
<212> PRT
<213> GHR chicken

<400> 9

Leu Trp Val Glu Phe Ile Glu Leu Asp Ile
1   5   10

<210> 10
<211> 10
<212> PRT
<213> prolactin receptor, human

<400> 10

Leu Leu Val Glu Tyr Leu Glu Val Asp Asp
1   5   10

<210> 11
<211> 10
<212> PRT
<213> prolactin receptor, rabbit, rat, mouse

<400> 11

Leu Leu Val Glu Phe Leu Glu Asn Asp Asp
1   5   10

<210> 12
<211> 10
<212> PRT
<213> Ca++ channel

<400> 12

Asp Asn Val Asp Tyr Leu Thr Arg Asp Trp
1   5   10

<210> 13
<211> 10
<212> PRT
<213> FGF Receptor Family

<400> 13

Gln Ala Ala Glu Tyr Leu Arg Ser Glu Thr
1   5   10

<210> 14
<211> 10
<212> PRT
<213> Transmembrane receptor sex precursor

<400> 14

Ile Asp Ala Glu Tyr Ile Ser Ala Glu Arg
1   5   10

<210> 15
<211> 10
<212> PRT
<213> IgE Receptor

<400> 15

Leu Lys Gly Glu Phe Ile Trp Val Asp Gly
1   5   10

<210> 16
<211> 10
<212> PRT
<213> ANGIOTENSIN CONVERTING ENZYME

<400> 16

Tyr Gly Ser Glu Tyr Ile Asn Leu Asp Gly
1   5   10

<210> 17
<211> 10
<212> PRT
<213> POTASSIUM CHANNEL IRK

<400> 17

Ser Glu Gly Glu Tyr Ile Pro Leu Asp Gln
1   5   10

<210> 18
<211> 10
<212> PRT
<213> PDGF RECEPTOR ALPHA-CHAIN

<400> 18

Asp Gly His Glu Tyr Ile Tyr Val Asp Pro
1 5 10

<210> 19
<211> 10
<212> PRT
<213> PDGF RECEPTOR BETA-CHAIN

<400> 19

Asp Gly His Glu Tyr Ile Tyr Val Asp Pro
1 5 10

<210> 20
<211> 10
<212> PRT
<213> Ca++ -channel

<400> 20

Asp Asn Phe Glu Tyr Leu Thr Arg Asp Ser
1 5 10

<210> 21
<211> 10
<212> PRT
<213> Cl- CHANNEL CLC7

<400> 21

Lys Ile Phe Glu Tyr Leu Arg Arg Asp Thr
1 5 10

<210> 22
<211> 10
<212> PRT
<213> TYROSINE-PROTEIN KINASE FRK

<400> 22

Ser Leu Gln Glu Tyr Leu Gln Asn Asp Thr
1 5 10

<210> 23

<211> 10
<212> PRT
<213> GLUT4 INS-REGULATED GLUCOSE TRANSPORTER

<400> 23

Thr Glu Leu Glu Tyr Leu Gly Pro Asp Glu
1 5 10

<210> 24
<211> 10
<212> PRT
<213> MHC-II(BETA) (RAT)

<400> 24

Asn Gln Glu Glu Tyr Leu Arg Tyr Asp Ser
1 5 10

<210> 25
<211> 10
<212> PRT
<213> ERB2 TKR

<400> 25

Glu Asn Pro Glu Tyr Leu Gly Leu Asp Val
1 5 10

<210> 26
<211> 10
<212> PRT
<213> ANION TRANSPORTER I

<400> 26

Arg Leu Lys Glu Tyr Leu Ala Gly Asp Val
1 5 10

<210> 27
<211> 10
<212> PRT
<213> VASCULAR ENDOTHELIAL GROWTH FACTOR

<400> 27

Leu Tyr Lys Asp Phe Leu Thr Leu Glu His
1 5 10

<210> 28
<211> 10

<212> PRT
<213> VASCULAR ENDOTHELIAL GROWTH FACTOR

<400> 28

Glu Gln Lys Glu Tyr Lys Ser Tyr Asp Ala
1 5 10

<210> 29
<211> 10
<212> PRT
<213> G PROTEIN-ACT. INWARD RECTIFIER K+-CHANNEL-1

<400> 29

Pro Glu Gly Glu Phe Leu Pro Leu Asp Gln
1 5 10

<210> 30
<211> 10
<212> PRT
<213> PROTEIN-TYROSINE PHOSPHATASE ZETA

<400> 30

Ser Asp Ser Glu Phe Leu Leu Pro Asp Thr
1 5 10

<210> 31
<211> 10
<212> PRT
<213> GLUTAMATE (NMDA) RECEPTOR SUBUNIT EPSILON 2

<400> 31

Ser Ala Leu Asp Phe Ile Arg Arg Glu Ser
1 5 10

<210> 32
<211> 10
<212> PRT
<213> RHESUS BLOOD GROUP-ASSOCIATED GLYCOPROTEIN

<400> 32

Ala His Asn Glu Tyr Leu Val Ser Glu Ile
1 5 10

<210> 33
<211> 10
<212> PRT

<213> DIHYDROPYRIDINE-SENSITIVE 1-TYPE

<400> 33

Val Thr Leu Asp Phe Leu Asp Ala Glu Leu
1 5 10

<210> 34
<211> 10
<212> PRT
<213> THROMBOPOIETIN RECEPTOR

<400> 34

Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu
1 5 10

<210> 35
<211> 10
<212> PRT
<213> SEROTONIN RECEPTOR 1B

<400> 35

Ser Ala Lys Asp Tyr Ile Tyr Gln Asp Ser
1 5 10

<210> 36
<211> 10
<212> PRT
<213> EPIDERMAL GROWTH FACTOR

<400> 36

Tyr Gln Gln Asp Phe Phe Pro Lys Glu Ala
1 5 10

<210> 37
<211> 10
<212> PRT
<213> SODIUM, CHLORIDE-DEPENDENT TRANSPORTER NTT4

<400> 37

Ser Lys Leu Gln Tyr Ile Leu Ala Gln Ile
1 5 10

<210> 38
<211> 10
<212> PRT
<213> RHODOPSIN

<400> 38

Thr Pro Leu Asn Tyr Ile Leu Leu Asn Leu
1 5 10

<210> 39
<211> 10
<212> PRT
<213> INTERLEUKIN-2 RECEPTOR BETA-CHAIN

<400> 39

Thr Ser Val Asp Leu Leu Asp Ile Asn Val
1 5 10

<210> 40
<211> 10
<212> PRT
<213> cAMP-DEPENDENT PROTEIN KINASE C, ALPHA, BETA

<400> 40

Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile
1 5 10

<210> 41
<211> 10
<212> PRT
<213> cAMP-DEPENDENT PROTEIN KINASE DELTA, EPSILON, GAMMA

<400> 41

Gly Thr Pro Glu Tyr Leu Ala Pro Glu Ile
1 5 10

<210> 42
<211> 10
<212> PRT
<213> SERINE/THREONINE KINASE PCTAIRE 1,2

<400> 42

Leu Val Phe Glu Tyr Leu Asp Lys Asp Leu
1 5 10

<210> 43
<211> 10
<212> PRT
<213> SERINE/THREONINE KINASE PCTAIRE 3

<400> 43

Leu Val Phe Glu Tyr Leu Asp Ser Asp Leu
1   5   10

<210> 44
<211> 10
<212> PRT
<213> SMALL GTP-BINDING PROTEIN Rab-7

<400> 44

Ile Gly Ala Asp Phe Leu Thr Lys Glu Val
1   5   10

<210> 45
<211> 10
<212> PRT
<213> SMALL GTP-BINDING PROTEIN Rab-9

<400> 45

Ile Gly Val Glu Phe Leu Asn Lys Asp Leu
1   5   10

<210> 46
<211> 10
<212> PRT
<213> SYNAPTOTAGMIN IV

<400> 46

Ile Ser Val Glu Phe Leu Val Leu Asp Ser
1   5   10

<210> 47
<211> 10
<212> PRT
<213> GLUTAMATE DECARBOXYLASE (GAD67)

<400> 47

Ser Asp Ile Asp Phe Leu Ile Glu Glu Ile
1   5   10

<210> 48
<211> 10
<212> PRT
<213> FRUCTOSE 1,6 DIPHOSPHATASE (FBPase)

<400> 48

Ala Ile Gly Glu Phe Ile Leu Val Asp Lys
1   5   10

<210> 49
<211> 10
<212> PRT
<213> CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

<400> 49

Gln Lys Gln Glu Tyr Lys Thr Leu Glu Tyr
1   5   10

<210> 50
<211> 5
<212> PRT
<213> EPITHELIAL Na+ CHANNEL

<220>
<221> X
<222> (4)..(4)
<223> X can be any amino acid

<400> 50

Pro Pro Pro Xaa Tyr
1   5